Molecular analysis and Phylogeography of Neotropical Amphibians

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When the mind is thinking it is talking to itself

Plato

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I. Acknowledgments

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II. Abstract

In this thesis, I comprised molecular and phylogeographic studies of neotropical frogs, presented in three chapters. The first chapter is focused on the molecular and acoustic analysis of the direct developing frog, *Ischcnocnema guentheri* (Brachycephalidae). This species is thought to have a widespread distribution range in the Brazilian Atlantic Forest (AF). Here I show that *I. guentheri* actually is a species complex composed of a minimum of six species. Two of these species were assigned to available names: *I. guentheri* and *I henselii*. The other four were defined as candidate new species. Their phylogeographical pattern partially agrees with paleo-models for the Atlantic Forest, but also suggests the existence of micro-refugia in less stable areas. *I. guentheri* sensu stricto, previously considered to be widespread, was found only in its type locality, a reserve within the urban area of Rio de Janeiro city. Until now, neither *I. guentheri* nor *I. henselii* has ben considered threatened according to IUCN (*International Union for Conservation of Nature*). However, my findings suggest, that the status has to be carefully re-evaluated in the next comprehensive update of the Red List of Brazil's amphibians.

The second study is about the molecular and biogeographic analysis of *Dendropsophus minutus*. Studies comprising the entire distribution of species that are assumed to occupy vast continental areas across political borders are rare, because data sampling is handicapped by financial, logistic and political factors. Consequently, taxonomic and phylogeographical studies are often focused on specific regions, whereas the entire range is studied rarely. Such is the case in *Dendropsophus minutus*, a putatively continentally distributed South American hylid. This species is still considered widespread despite regional morphological and biacustical differences. To analyze the molecular diversity and the phylogeographical pattern throughout the range of the species, I made a collaborative effort bringing together 416 tissue samples representing almost the entire distribution of the species. We found more than 19 largely allopatric deep mitochondrial lineages within *D. minutus*, most of them with high node support in the phylogenetic tree inference. Ten of these lineages form a monophyletic clade which I define as the *D. minutus* complex. The other lineages represent related species that together with the *D. minutus* complex form the *D. minutus* group. My results support an Amazonian origin of the *D. minutus* group with a subsequent dispersal to eastern Brazil and Atlantic Forest where the *D. minutus* complex originates. The phylogeographical pattern of the *D. minutus* complex largely agrees with previous molecular studies of the AF fauna.

The third chapter presents a comparative analysis of nine AF species. The AF is a species-rich tropical region that contains a high level of endemism. In recent years, attention has been given to this tropical area and scientists have been proposing different diversification mechanism to explain its diversity pattern. One of the most debated hypotheses is the Refugia Model (RM). Molecular studies have shown evidence both for rejecting and supporting the hypothesis. One aspect that weakens the RM is the existence of high genetic diversity in some populations distributed in areas that were supposed to contain low genetic diversity according to the model. This evidence suggests the existence of additional stable areas in the AF besides the ones currently proposed. Comparative phylogeography has the potential to uncover the existence of a common biogeographic history among co-distributed taxa. In order to perform a comparative analysis of AF species, I included published mitochondrial sequences from six well-sampled AF species: five amphibians and one snake. Additionally I have generated sequence data for three other co-distributed frog species to be included in the analysis. For the comparative analysis, I used a method recently implemented in the software BEAST 1.7 that can estimate the geographical origin of nodes of a genealogy. The results show that deep mitochondrial sister lineages have originated apart from each other and away from contact zones. Furthermore, I found high overlapping frequency of center of origins of co-distributed lineages in two regions of the studied area. One region is located at the northern centre of the AF, which coincides with the recently proposed RM. The other center of origin is in the southeastern AF, which agrees with floristic studies proposing a refugium in this region.

III. Zusammenfassung

In dieser Dissertation habe ich in drei Kapiteln die molekularen und phylogeographischen Arbeiten an neotropischen Fröschen zusammengestellt. Das erste Kapitel befasst sich mit der molekularen und akustischen Untersuchung von der sich direkt entwickelnden Froschart *Ischnocnema guentheri* (Brachycephalidae). Bei dieser Art wurde davon ausgegangen, dass sie ein sehr grosses Verbreitungsgebiet im brasilianischem Atlantischem Regenwald (AR) hat. Ich habe herausgefunden, dass *I. guentheri* ein Artenkomplex ist, der aus mindestens sechs Arten besteht. Zwei dieser Gruppen habe ich bereits beschriebenen Arten zugeordnet, nämlich *I. guentheri* und *I. henselii*. Die anderen vier sind voraussichtlich neue Arten. Ihr phylogeographisches Muster stimmt zum Teil mit Paleoklimatischen Modellierungen für den AR überein, aber lässt auch vermuten, dass Mikrorefugien in weniger stabilen Gegenden existiert haben. Die Art *I. guentheri* (im engeren Sinne), von der bisher vermutet wurde, dass sie ein großes Verbreitungsgebiet hat, habe ich nur in ihrem Typusgebiet entdecken können, nämlich einem Schutzgebiet in einer urbanen Gegend der Stadt Rio de Janeiro. Obwohl bisher weder von *I. guentheri* noch von *I. henselii* vermutet wurde, dass sie vom Aussterben bedroht sind, empfehle ich, dass der Bedrohungszustand gemäß IUCN (Internationale Vereinigung für die Bewahrung der Natur und natürlicher Ressourcen) bei der nächsten Überarbeitung der Roten Liste von Brasiliens Amphibien noch einmal überprüft werden sollte.

Der zweite Teil meiner Dissertation befasst sich mit der molekularen und bioakustischen Untersuchung von *D. minutus*. Bisher gibt es nur wenige Untersuchungen an Arten, die das gesamte Verbreitungsgebiet berücksichtigen, besonders wenn es sich über eine großes Areal und über politische Grenzen hinweg erstreckt, da die Probennahme durch finanzielle, logistische und politische Faktoren erschwert wird. Das hat zur Folge, dass sich taxonomische und phylogeographische Untersuchungen oft nur auf einen Teil eines Verbreitungsgebietes beziehen. Dieses ist beispielsweise der Fall bei *D. minutus*, einem vermeintlich sehr weit verbreiteten südamerikanischen Hyliden. Die Art ist sehr weit verbreitet, obwohl es regional morphologische und bioakustische Unterschiede gibt. Um die molekulare Diversität und phylogeographischen Muster aus dem gesamten Verbreitungsgebiet zu untersuchen, habe ich mit der Unterstützung anderer Wissenschaftler 416 Gewebeproben aus zumindest fast dem gesamten Verbreitungsgebiet zusammenstellen können. Ich habe mehr als 19 allopatrische sehr unterschiedliche mitochondriale Linien innerhalb von *D. minutus* gefunden, bei denen die Mehrheit der Knotenpunkte des phylogentischen Baums statistisch stark abgesichert ist. Zehn dieser Linien gehören zu einer monophyletischen Klade, die ich als den *D. minutus* Komplex bezeichne. Die anderen Linien gehören zu verwandten Arten, die zusammen mit dem *D. minutus* Komplex die *D. minutus* Gruppe ausmachen. Meine Ergebnisse unterstützen, dass der Ursprung der *D. minutus* Gruppe im Amazonasgebiet liegt, mit einer darauffolgenden Ausbreitung nach Ostbrasilien und in den AR, in dem wiederum der *D. minutus* Komplex seinen Ursprung hat. Das phylogeographische Muster des *D. minutus* Komplexes stimmt größtenteils mit dem anderer molekularen Untersuchungen der Fauna des Atlantischen Regenwaldes überein.

Das dritte Kapitel beinhaltet eine vergleichende Untersuchung von insgesamt neun Arten aus dem AR. Dieser ist ein artenreiches tropisches Gebiet, das einen hohen Grad an Endemismus aufweist. Seit einigen Jahren wird diesem Gebiet viel Aufmerksamkeit erteilt und Wissenschaftler haben verschiedene Mechanismen vorgeschlagen, die die Diversitätsmuster erklären sollen. Eines der am meisten diskutierten Modelle ist das Refugialmodell (RM), das von den Ergebnissen einiger molekularer Arbeiten unterstützt wird aber von anderen wiederum nicht. Ein Aspekt der das RM schwächt ist der, dass in einigen Gebieten Populationen mit hoher genetischer Diversität gefunden wurden, die aber dem Modell nach nur wenig genetische Variabilität enthalten sollten. Dieses kann als Beweis dafür interpretiert werden, dass es zusätzlich zu den bekannten Gebieten auch noch weitere klimatisch stabile Gegenden gegeben hat. Vergleichende phylogeographische Untersuchungen ermöglichen es, bei Arten mit ähnlicher Verbreitung, eine eventuelle gemeinsame biogeographische Geschichte aufzudecken. Um eine solche vergleichende Untersuchung von Arten aus dem AR durchzuführen, habe ich bereits veröffentlichte mitochondriale Sequenzen von sechs gut untersuchten Arten aus dem AR verwendet, nämlich von fünf Froscharten und einer Schlange. Zusätzlich habe ich für diese Analyse Sequenzdaten für drei weitere Froscharten mit demselben Verbreitungsgebiet generiert. Für diese vergleichende Analyse habe ich eine kürzlich veröffentlichte Methode, integriert in der Software BEAST 1.7, verwendet, mit der man den geographischen Ursprung für Verzweigungsknoten einer Genealogie abschätzen kann. Die Ergebnisse zeigen, dass alte mitochondriale Schwestergruppen ihren Ursprung in unterschiedlichen Gebieten hatten, der auch nicht in der Nähe heutiger Kontaktzonen liegt. Des Weiteren habe ich eine große Übereinstimmung der ermittelten wahrscheinlichen Ursprunggebiete bei verschiedenen Linien mit heute ähnlichen Verbreitungsgebieten gefunden. Das eine Gebiet befindet sich im nördlichen Zentrum des AR, und stimmt mit dem kürzlich vorgeschlagenen RM überein. Das andere Ursprungsgebiet befindet sich im südöstlichen AR und stimmt mit dem Refugium überein, das bei floristischen Untersuchungen ermittelt wurde.

IV. Preface

Dear reader, in this thesis you will find part of the work I have carried out during my PhD studies at the Evolutionary Department of the Technische Universität Braunschweig from October 2009 to September 2012. The work presented here is divided in three chapters, all of them regarding the molecular analysis and biogeography of Neotropical amphibians. The first two chapters have both a taxonomic and biogeographic perspective while the third chapter is focused on biogeography only. The species analyzed are either widely distributed in South America (Chapter 2) or restricted to the Brazilian east coast and its associated rain forest, the Brazilian Atlantic Forest (AF) (Chapters 1 and 3). During my studies I had the opportunity to lead the writing and analysis of the work that culminated in this thesis and, in addition, I could fortunately count on the help of many researchers. Besides the important supervision of Dr. Miguel Vences, I had the support of Dr. Célio Haddad and his amphibian collection. This allowed me to perform preliminary analysis on previously collected samples, to guide the planning of the field trips. Those were then carried out during austral summer of 2010-2011, and were optimized to fulfill a comprehensive sampling of each species' distribution (rather than exploring new areas). Additionally, new collaborations could also be established in order to get a more extensive sampling and adequate analysis. In this preface I will give a brief introduction to the content of each chapter, highlighting important collaborations that improved the work, as well as describe the methodological approach used in each chapter and the theoretical background related to it.

Chapter 1: From Widespread to microendemic: molecular and acoustic analyses show that *Ischnocnema guentheri* is endemic to Rio de Janeiro, Brazil.

This chapter depicts the molecular and acoustic analysis of the *Ischnocnema guentheri* species complex (**Figure 1**). This species is supposed to have a widespread distribution in the Atlantic Forest (**Figure 2**). However, evidence from advertisement call variation suggests that *I. guentheri* is in fact a species complex. In order to have a better understanding of how many species are present in this complex and what is the distribution ranges of each of them, we analyzed different sources of data (mitochondrial DNA, nuclear DNA and bioacoustics) which were combined in an integrative approach.

Figure 1 Picture of *Ischnocnema guentheri* (photo by the author)

The integrative taxonomy (Dayrat, 2005; Padial et al., 2010; Will et al., 2005), is a term coined to bring the idea of integrating evidence from different fields of biology for species delimitation. In this sense the word *integrative* contains in itself the notion of multidiciplinarity in a close relationship with *Integrative Biology*. In the words of Dr. Marvalee Wake (Wake, 2003) Integrative Biology has an "*emphasis on organismal biology from multiple perspectives in an attempt to evaluate issues of biological complexity and their many dimensions"*. In my point of view the idea of a multidisciplinary taxonomy is actually an inevitable result of a conceptual change of species definition, mainly switching from a static to a dynamic understanding of the species unit. When we understand that species are not a static unit but rather a changing unit, we realize that a species delimitation based in a single line of evidence will likely lead to an erroneous species hypothesis (de Queiroz, 1998; de Queiroz, 2007). In this way the integrative taxonomy goes in the same direction of the *New Systematics* described by Mayr (1942). In fact the term *New Systematics* was first used by Hubbs, (1934) and further introduced by Huxley (1940), but also adopted and described by Mayr (1942). In his groundbreaking book *Sytematics and the Origin of Species from the viewpoint of a Zoologist* Dr.Ernst Mayr in his view of the New Systematics writes that "*…the purely morphological species definition has been replaced by a biological one, which takes ecological, geographical, genetic and other factors into consideration…*". It is important to mention that Mayr considered Systematics and

Taxonomy *"as approximate synonymous"****.** It is also worth to consider that the *"biological one"* for Mayr was very likely linked to his Biological Species Concept. Although the biological species concept had been in a manner relaxed in order to accommodate non "biological" species (Grant and Grant, 1992; Pfennig, 2007; Veen et al., 2001), it seems that with his species concept Mayr was trying to grasp and integrate many dimensions of an organism in a species definition. Thus, I would consider that the idea of integrating started to arise with the "*Modern Evolutionary Synthesis*", approximately 100 years after Darwin's *On the origin of species*, when the understanding of what a species is and how they arise began to be clarified by bringing evolutionary thinking to the taxonomic work. However, what seemed to be an improvement on Taxonomy created a crisis in the field, as highlighted by Wheeler (2008), in his introductory chapter of the book *The New Taxonomy*. However Wheeler does not see the *New Sytematics*, supported by Huxley and Mayr, as a good contribution to Taxonomy. Instead, he seriously criticizes it blaming Huxley as providing *"the battle cry for those who would dilute, detract from and eventually decimate taxonomy"*, further he continues, *"rather than recognizing the need for a new science associated with mutation, selection and shifting gene frequencies – that is, the study of the process of speciation, Huxley and others tried to redefine the existing science of taxonomy to meet this need"*. Dr. Quentin Wheeler advocates that with the Modern Synthesis the introduction of population thinking into Taxonomy mixed up process with patterns and that population genetics and taxonomy are incompatible: *"it is necessary to separate processes from patterns in order to do justice to either*". Most biologists would agree that species are separately evolving metapopulations. So, excluding population thinking seems retroceding in time and disintegrating instead of integrating. Interestingly, Wheeler himself supports the Integrative Taxonomy (Will et al., 2005). Nevertheless, it seems that Wheeler considers taxonomy as the purely work of finding patterns and giving name to biological hierarchies.

***** Taxonomy and Systematics are both junctions of Greek words that in the end have the same meaning. Systematics is a union of *sietemium*, wich means arrangement*;* and *atics*, which means science. Taxonomy is a union of *taxis*, which means arrangement; and *nomia* which means method. When the scientific method is used to arrange the biological information Taxonomy and Systematics become different words to express the same idea.

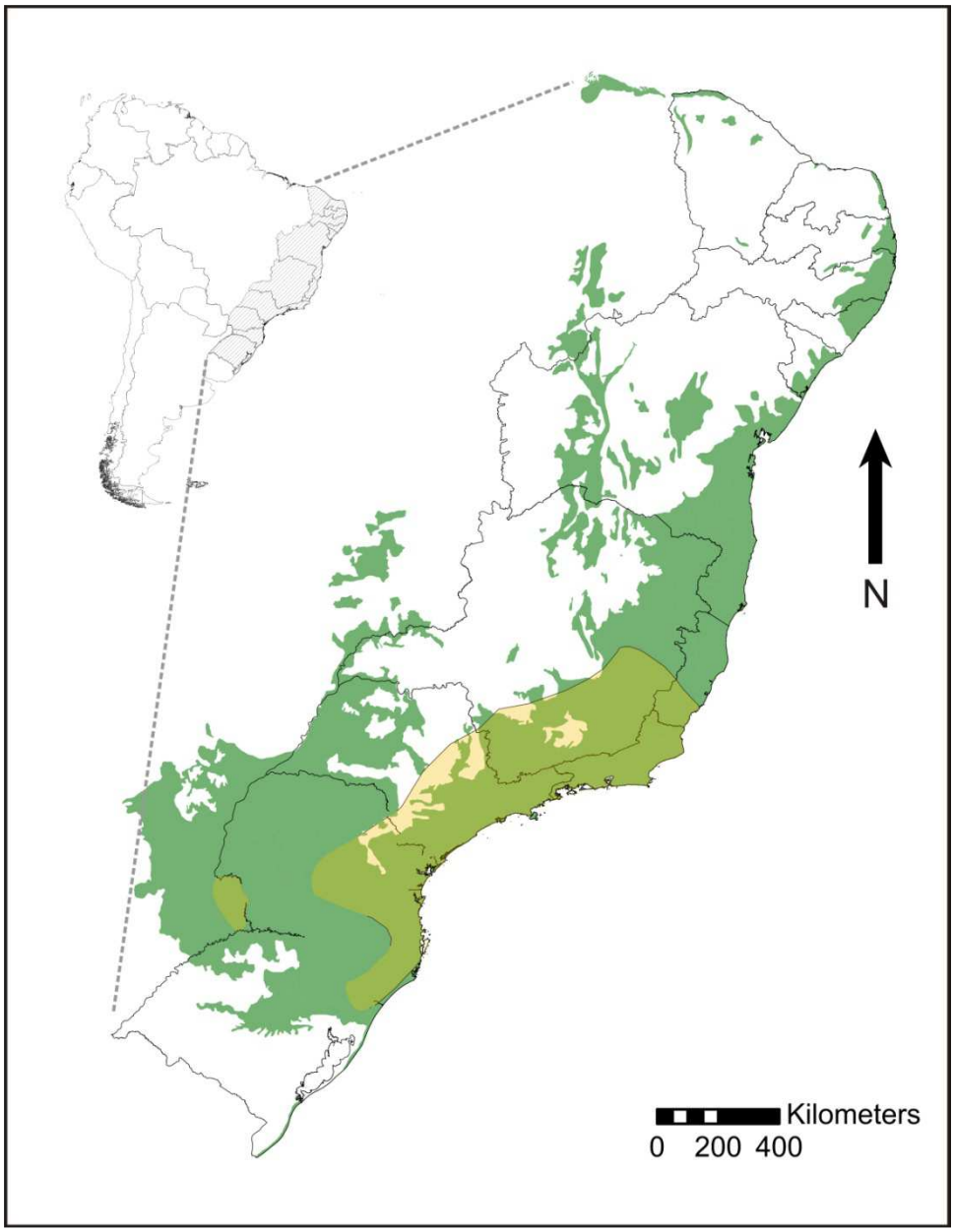

Figure 2: Distribution range of *Ischnocnema guenthei* according to the IUCN (pale yellow). Data dowloaded on 13.09.2012 from www.iucnredlist.org. The green layer represents the original range of the Atlantic Forest (all biomes combined).

Many biologists will agree with Wheeler, many will not, including me. I see biological patterns as "snap shots" of processes; hence, both are necessarily linked. Maybe, the most important cause of such crisis is that *"many non-taxonomist biologists are now frustrated by the 'old-fashioned' typological approach with which taxonomists still describe species"*(Dayrat, 2005). Because the biological information is complex and dynamic, organizing and understanding it demands a case by case analysis, which means that it is virtually impossible to create a simple universal rule for taxonomy. The lack of a clear rule let the work of taxonomy open to subjectivity. So, taxonomists are in some extent free to do taxonomy in their own way. That is why it still possible to find Linnean, typological

species delimitation, which wont sound, particularly to non-taxonomists. However, the impossibility of creating a universal rule for taxonomy does not mean impossibility of creating criteria of how to do taxonomy. In this way, the Integrative Taxonomy, if truly integrative (Padial and De La Riva, 2010), is an elegant way to overcome this aspect of the taxonomy crisis. By integrating methods from different areas of biology, taxonomy becomes a form of Integrative Biology and consequently it turns into a much powerful tool for the understanding of biological complexity. Inevitably, it becomes more interesting, more informative and more reliable.

Following an integrative approach we found that the *I. guentheri* complex is composed by a minimum of six species. We also provide evidence that support the hypothesis that *Ischnocnema guentheri* is endemic to its type locality, the city of Rio de Janeiro, Brazil.

Chapter 2: Do continentally widespread species of frogs exist in the Neotropics? Molecular analyses indicate that *Dendropsophus minutus* is a lineage-rich and biogeographically complex taxon.

In this study we performed a molecular and biogeographical analysis of *Dendropsophus minutus* (**Figure 3**). Our analysis reveals high diversity and many deep mitochondrial lineages within *D. minutus* distribution, supporting the hypothesis of a species complex. This species was already suggested to be a species complex by many herpetologists. However because all analysis so far were restricted to certain parts of its distribution it continued to be considered continentally widespread (**Figure 4**). One interesting aspect of this second chapter is the geographical coverage of the analyzed samples, which represent virtually the entire distribution of the species. This sampling was achieved with the establishment of a collaboration among over 30 scientists. Most of the laboratory work was carried out by me in the TU-Braunschweig. I was also the responsible for analyzing the data and leading the manuscript writing. The fact that this investigation brings together so many herpetologists makes it an example of collaborative work to be followed in future studies of putatively widespread amphibians, particularly in cases where the species range overcome political borders. There are other cases of supposedly widespread neotropical amphibians, like *Rhinella margaritifera* (Bufonidae); *Scinax ruber* and *Trachycephalus typhonius* (Hylidae); and *Leptodactylus fuscus* (Leptodactylidae), that have been suggested as likely containing several species under a single name. In addition to the taxonomic importance the molecular and phylogeographical analysis of such widespread species are important for a better understanding of the biogeography of South America, given that a continental scale phylogeography, as opposed to a regional scale phylogeography, has the potential to provide a more comprehensive view of the biogeography of South America.

Figure 3: Picture of *Dendropsophus minutus* (photo by the author)

It is clear that tropical regions contain most of the biodiversity of the planet (Myers et al., 2000). However, molecular studies show that the number of tropical amphibian species are likely underestimated, and cryptic diversity might be hidden under supposedly widespread species (Fouquet et al., 2007a; Funk et al., 2012) (Chapter 1). A combination of factors can cause such underestimation. First, amphibian morphology is extremely conserved when compared to other vertebrates (Cherry et al., 1978; Cherry et al., 1977; Emerson, 1988; Shubin and Jenkins, 1995), and morphological convergence can be identified in several amphibian groups (Bossuyt and Milinkovitch, 2000; Mueller et al., 2004; Parra-Olea and Wake, 2001; Wake, 1991). This makes the analysis of other source of information, like molecular data and ecological parameters (advertisement call) of extreme importance for amphibian taxonomy. Second, the tropics are poorly studied, especially in terms of molecular analysis when compared to other regions of the planet (Beheregaray, 2008). Thus, the number of species of frogs is certainly underestimated in the tropics. Our analysis supports this idea by demonstrating a huge genetic diversity under the name *D. minutus*. Nevertheless, other sources of data are still needed and therefore we refrain from making conclusive statements about the exact number of species present in this complex. However, we provide the first step for the taxonomic revision of *D. minutus.* Such a revision can also have great impacts on conservation because the discovery of cryptic diversity, splitting once widely distributed species into several species, each with a significantly

smaller range, may easily change the status of certain populations from 'least concern' to one of the various threat categories defined by IUCN red list criteria.

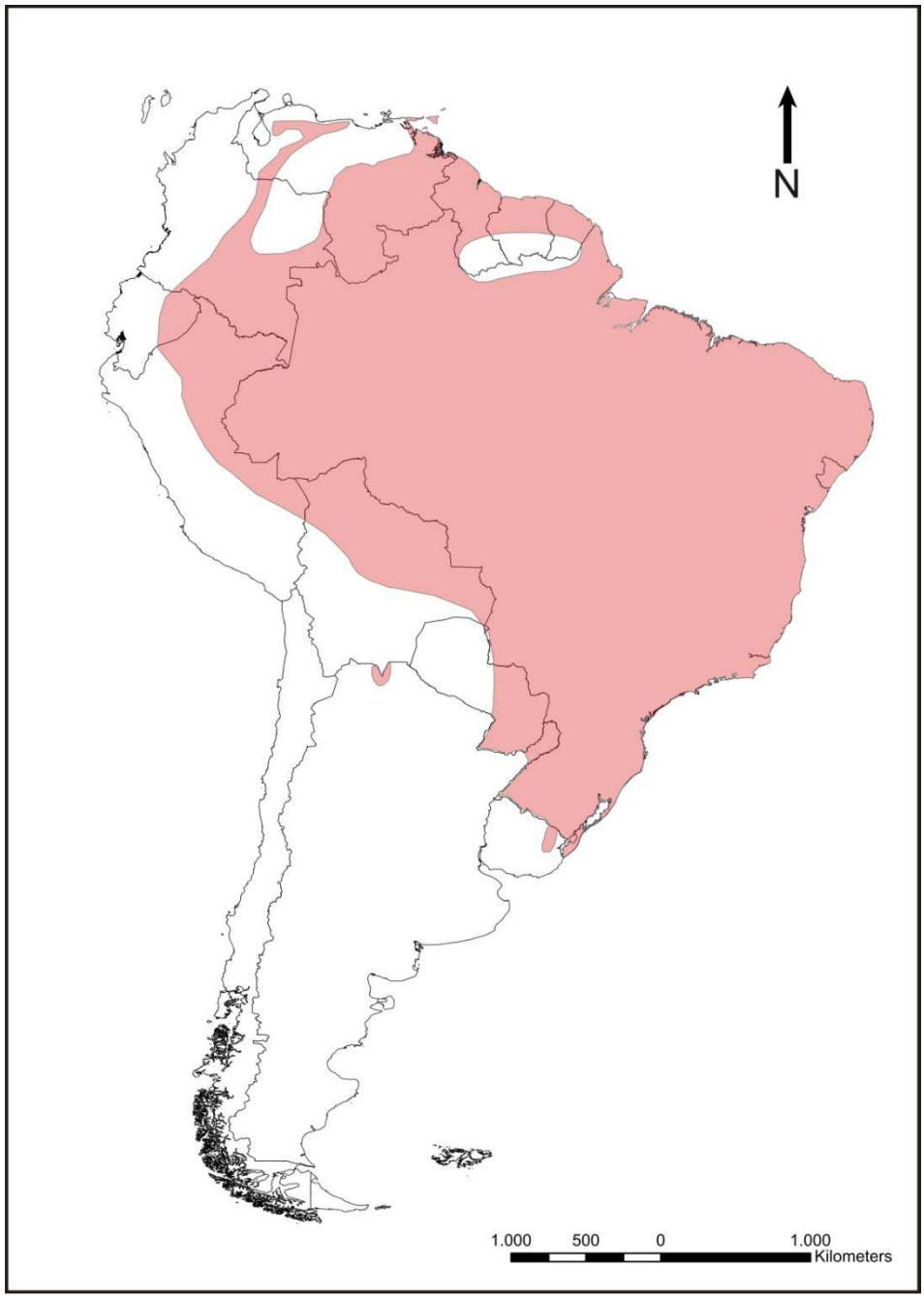

Figure 4: Distribution range of *Dendrpsophus minutus* according to the IUCN. Data downloaded on 13.09.2012 from www.iucnredlist.org.

On the biogeographic point of view the analysis of *D.minutus* can give new insights into the biogeography of South American amphibians. As most Neotropical amphibian species are distributed within certain biomes, phylogeographical studies are often focused on specific regions whereas the global continental picture is scarcely studied (Crawford and Smith, 2005; Elmer et al., 2007; Noonan and Gaucher, 2005; Prado et al., 2012; Symula et al., 2003; Thomé et al., 2010). Interestingly, because

of the wide distribution of the nominal *D. minutus*, a phylogeographic study has the potential to integrate regional and continental scale analyses. For instance, phylogeographical patterns from different regions of the continent can be analyzed simultaneously and further compared with previous studies, while the global continental pattern can be assessed. To analyze the biogeography of *D. minutus* we applied an interesting method of phylogeographical reconstruction. The outcome of this analysis show that *D.minutus* and close related species has a central Amazonian origin, from there the group subsequently dispersed to other regions of South America. The results also suggest a southern dispersal route between Amazonia and the Brazilian Atlantic Forest and more than one migration event between these two regions. This manuscript will be submitted to the journal Proceedings of the Royal Society B.

Chapter 3: Finding stable areas with genes: comparative phylogeography of Brazilian Atlantic Forest species

In this chapter I performed a comparative analysis of nine Atlantic Forest species, eight amphibians and one snake (**Figure 5**). My idea was to use the same phylogeographic method used in the second chapter to compare the phylogeographical patterns of eight amphibians and one reptile. The aim was mainly to check if the phylogeographical patterns would support the Refugia Model proposed to explain the biogeography of the Brazilian Atlantic Forest (Carnaval et al., 2009; Carnaval and Moritz, 2008).

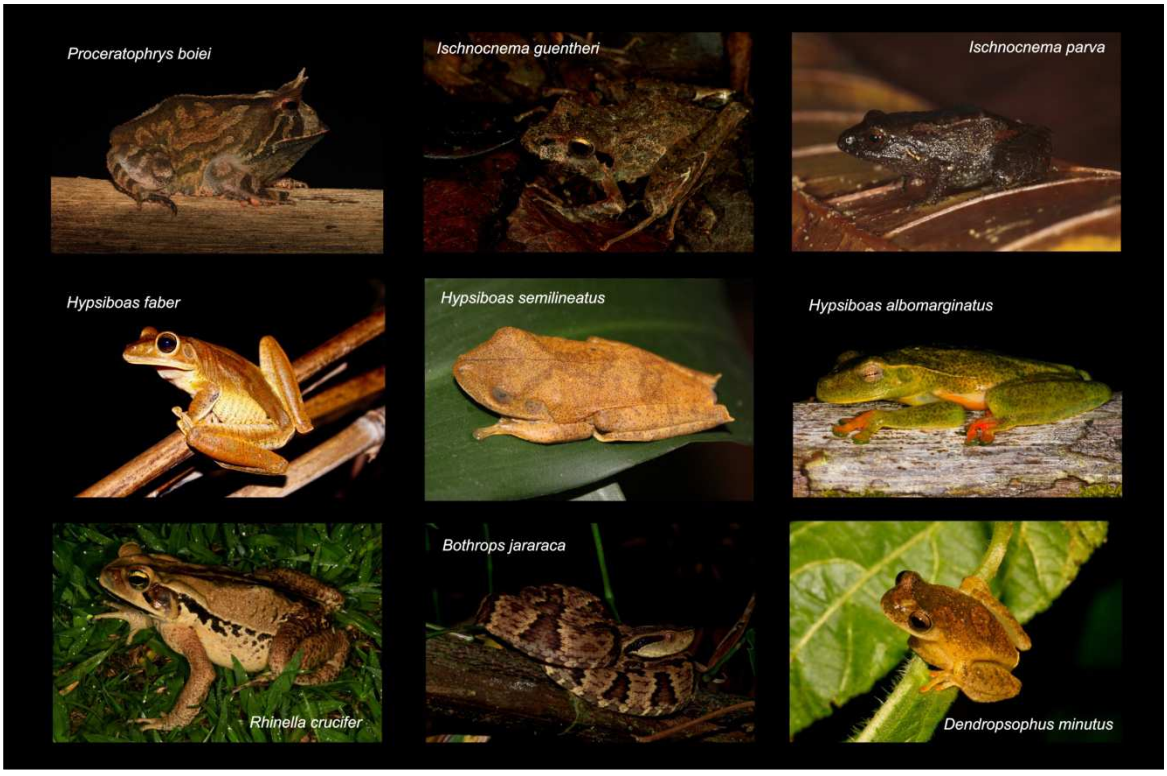

Figure 5: Pictures of species analyzed in the third chapter (all photos by the author)

The approach currently used by scientists to identify refugia and to propose the Refugia Model is based on distribution modeling (Graham et al., 2006), which are confronted with genetic data to check for congruency. We should expect a great amount of uncertainty in these modeled refuges because they are based on current distribution models projected to past climates scenarios, which can be different depending on the method used to generate them. As a consequence, one can obtain different past suitability areas for the same species depending on the past climate scenario to which the current model is projected (e.g. Amaro et al. 2012). As an attempt to minimize this uncertainty scientists have adopted the approach of overlapping different past distribution models to check for congruency among them. These models are further overlapped with current distribution models and the regions where all models overlap are considered for generating hypothesis of stability (e.g. Thomé et al 2010; Carnaval et al. 2009). To check for support the hypothesis is compared with genetic data, most of the time in a subjective way (e.g. Thomé et al. 2010; Amaro et al 2012). The only objective way used so far to test refuge hypotheses is the Hierarchical Aproximate Bayesian Computation (HABC) method implemented in the program msBayes (Hickerson et al., 2007; Huang et al., 2011). In the single example of the use of this approach for the Atlantic Forest the hypothesis of stability and refuges was supported (Carnaval et al., 2009). Despite the power of model based inference, with the use of Approximate Bayesian Computation (ABC) for hypothesis testing of complex evolutionary scenarios (Beaumont et al., 2010; Fagundes et al., 2007) the method is still largely inaccessible to non specialists because of the complex implementation of the computations involved. The only software having user friendly interface which can facilitate its use is the DIYABC (Cornuet et al., 2008). Moreover, in most of the ABC softwares there is a lack in of statistical methods to evaluate type one and type two errors (Cornuet et al., 2010), which is considered a caveat of this type of analysis. Then, the implementation of a simpler full-likelihood method that can be used to check for congruence between distribution modeling and phylogeographical patterns would be highly beneficial.

The continuous phylogeographical analysis recently implemented in the software Beast 1.7 (Drummond et al., 2012) can be used to estimate the geographical origin of a particular genealogy. If a certain species had in the past its distribution restricted to a refugium, this refugium would be the center of origin of the current distribution of the species, which in principle can be identified by estimating the center of origin of a particular genealogy (Lemmon and Lemmon, 2008). However, to support a common refugium, centers of origin of co-distributed taxa should overlap. Thus, in theory it is possible to identify stable areas using only genetic data. The phylogeographical method is easy to implement being independent of substitution rate and demographic parameters. The advantage is that the phylogeographical pattern can be objectively confronted with distribution models; also, it would provide an objective way to compare phylogeographical patterns of co-distributed species. The results presented in the third chapter show a striking overlapping pattern of centers of origins that is

congruent with the Refugia Model, supporting two diversification centers within the studied area. This chapter is a preliminary manuscript that will submitted for publication after final refinement.

V. From widespread to microendemic: molecular and acoustic analyses show that *Ischnocnema guentheri* (Anura: Brachycephalidae) is endemic to Rio de Janeiro, Brazil

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Abstract

Especially in tropical amphibians, many species are restricted to very small ranges, and this microendemism coupled with ongoing habitat loss and susceptibility to emerging pathogens imperils the long-term persistence of these species. Incomplete taxonomic and distributional knowledge might obscure conservation assessment, especially in putatively widespread species that are typically considered to be of Least Concern in Red List assessments, but that in fact may constitute complexes of partly microendemic species. Such is the case of *Ischnocnema guentheri* which together with the recently recognized *Ischnocnema henselii* is thought to occupy most of Brazilian Atlantic Forest. To test whether these taxa might constitute a species complex of range-restricted and thus potentially threatened species, we analyzed 168 samples assigned to this species complex for two molecular markers, 16S rRNA (16S) and Recombination activation gene 1 (RAG1). Closely related species were also included in the 16S analysis in order to verify the monophyly of the complex. Congruent evidence from the molecular data and from analyses of advertisement calls support the existence of six distinct species within the complex: *I. guentheri* and *I. henselii* as well as four candidates to new species (CS). The lineages are distributed as a mosaic in the Atlantic Forest and are sympatric at some localities without indication of admixture. Their phylogeographical pattern partially agrees with paleo-models for the Atlantic Forest, but also suggests the existence of micro-refugia in less stable areas. *I. guentheri*, previously considered to be widespread was only found in its type locality, a reserve within the urban area of Rio de Janeiro city. Although none of the species studied appears highly threatened with extinction, we recommend their IUCN threat status be careful re-evaluated in the next comprehensive update of the Red List of Brazil's amphibians.

Key Words: IUCN threat categories; Integrative taxonomy, Terrarana, *I. guentheri*, *I. henselii*, Brazilian Atlantic Forest.

V.I. Introduction

Amphibians are a highly threatened group of vertebrates due to a combination of mainly habitat destruction and the action of pathogens, and might be seen as indicators of an ongoing human-induced sixth mass extinction on Earth (Wake and Vredenburg, 2008). The Global Amphibian Assessment GAA (Stuart et al., 2004)first reviewed amphibians at a global scale following IUCN criteria (IUCN 2001), and flagged 427 species (7.4%) being at the brink of extinction in the Critically Endangered category, although only about 150 can be considered as extinct or possibly extinct. Given that for many tropical amphibian species there are very few data on actual population density and population trends, the most important variables used to assess the threat status of a species (Stuart et al., 2004) have been the size of its geographical range and its habitat requirements, combined with the rate of habitat loss within its range. For a reliable threat assessment it is therefore crucial that the taxonomy and the distribution range of species are known, and it is mainly due to the lack of such knowledge that the GAA listed 1294 species (22.5%) to be Data Deficient (Stuart et al., 2004). In a scenario of species decline in combination with evidence of undescribed diversity, taxonomic efficiency thus becomes crucial given that species are the currency of biological conservation (Andreone et al., 2008; Fouquet et al., 2007a; Myers et al., 2000; Stuart et al., 2004).

The advance of molecular techniques has changed the way we access biological diversity of amphibians (Fouquet et al., 2007a; Meegaskumbura et al., 2002; Vieites et al., 2009). In combination with other tools and with increased exploration of tropical areas of hyper-diverse biotas, increased use of DNA sequences as taxonomic character has boosted species description and improved species delimitation (James, 1999; Köhler et al., 2005). The understanding of species in an evolutionary framework brings taxonomy to a new multidisciplinary level. If species are understood as separately evolving metapopulations, any species delimitation based on uncritical application of a single line of evidence can be misleading, and independent characters can be incongruent because they will differentiate at different times of the speciation process (de Queiroz, 1998; de Queiroz, 2007). Integrative taxonomy, which relies on multidisciplinarity, comes as an attempt to identify species more accurately, because the reliability of delimiting independent evolving lineages increases by combining independent information (Dayrat, 2005; Padial et al., 2010).

Direct developing frogs of the Terrarana clade (Hegdes et al., 2008) are characterized by a high species diversity and remarkable morphological stasis among many closely related species, and morphological convergence among major subclades. At the intraspecific level, several studies have found deep genetic divergence between mitochondrial lineages which are difficult to access through morphology (Crawford, 2003; Crawford and Smith, 2005; Elmer et al., 2007; Padial and De la Riva, 2009; Rodriguez et al., 2010; Streicher et al., 2009; Velo-Anton et al., 2007; Wang et al., 2008).

The target species of the present study, *Ischnocnema guentheri* Steindachner 1864, is a medium-sized terraranan frog supposed to range-widely in the Atlantic Forest of Brazil, being the most widespread species of the Brachycephalidae (Hegdes et al., 2008; Heyer, 1984). Because of this wide range that encompasses numerous protected areas, the species is included in the Least Concern category of the IUCN Red List (IUCN 2011). However, preliminary data have indicated a puzzling taxonomic situation within this widespread species. Already in the original species description a high level of phenotypic variation was reported (Cochran, 1955), and in the most comprehensive revision to date, Heyer (1984) confirmed the existence of this extensive morphological variation within *I. guentheri.* In a subsequent study, (Kwet and Solé, 2005) resurrected *Hylodes henselii* Peters 1870, from the synonymy of *I. guentheri* on the basis of advertisement call differences. These authors also demonstrated the existence of call variation within populations that remained included in *I. guentheri* , suggesting a still unresolved species complex. Thus, *I. guentheri* is considered a widespread species despite its remarkable morphological and bioacoustic diversity. Its current distribution ranges across the Atlantic Forest, from Espírito Santo state to Santa Catarina state (Heyer 1984; Kwet and Solé 2005).

Resolving the taxonomy of such problematic and widespread taxa requires a combination of approaches and characters. Such revisions can be rendered most efficient using as primary core evidence of molecular markers of fast lineage sorting, i.e. mitochondrial DNA (mtDNA) sequences, with a subsequent quest for congruence of deep mtDNA lineages with geographical, morphological and bioacoustic evidence (Padial et al. 2010). The use of bio-acoustical data is critical to support species hypotheses in anurans as sexual selection can lead to rapid divergence of advertisement calls and thereby generate prezygotic reproductive barriers (summary inVences and Wake, 2007). Here we follow this approach and combine mtDNA evidence with nuclear DNA sequences and preliminary bioacoustic data in samples covering almost the entire known distribution of *I. guentheri* and *I. henseli*, as a case study of how improved taxonomic resolution might impact threat assessment in putatively widespread species of direct-developing frogs. Our results support the species complex hypothesis and identify a minimum of four candidate species besides the two described species, with syntopic occurrence of at least three of these species. We find *I. guentheri* being restricted to the immediate surroundings of its type locality in an isolated forest in Rio de Janeiro city, exemplifying that putatively widespread amphibian species can be in fact microendemic and more susceptible to be categorized within a threatened Red List category.

V.II. Methods

V.II.a. Laboratory Methods

We analysed 161 samples identified as *Ischnocnema guentheri* and *Ischnocnema* cf. *guentheri* deposited in the tissue collection of Célio F.B. Haddad (CFBH-T) at the Universidade Estadual Paulista – UNESP, Rio Claro, or collected during field work from November, 2010 to January, 2011; plus seven samples identified as *Ischnocnema henselii* from Muzeu de Ciências da Pontifícia Universidade Católica do Rio Grande do Sul (MCP) for part of the 16S gene. Part of those samples (102) was also analyzed for the RAG1 gene. The laboratory procedures were performed at the Zoological Institute of TU Braunwschweig, Germany. All samples were transported to Germany in accordance with the respective German and Brazilian laws. Genomic DNA extractions were carried out following a standard salt extraction protocol (Bruford et al., 1992). Fragments of mitochondrial 16S rDNA and of the recombination activating gene 1 (RAG1), were obtained via polymerase chain reaction (PCR) using the following primers: 16S-IschF2 5'- AAAAAGAAGGAACTCGGCAAA, 16S-IschR1 5'- CCTGATCCAACATCGAGGTCGT, developed is this study, and AmpF2 and AmpR1 from Chiari et al. (2004), respectively. Reactions were performed in a final volume of 12.5 µl using the following concentration of reagents: 0.24 µM of each primer, 200 µM of dNTP, 1xPCR buffer, and 0.4 units of GoTaq DNA polymerase (Promega, Mannheim, Germany). PCR products were cleaned with enzymatic purification: 0.15 units of Shrimp Alkaline Phosphatase (SAP) and 1 unit of Exonuclease I (New England Biolabs, Frankfurt am Main, Germany) incubated for 15 min at 37°C followed by 15 min at 80°C. Purified PCR products were sequenced on an automated DNA sequencer (Applied Biosystems ABI 3130XL). Sequencing reaction (10µl) contained 0.2 or 0.3 µl of PCR product 0.5µl of BigDye 3.1 (Applied Biosystems, Darmstadt, Germany) and 0.3 µM of primer. The mitochondrial fragments were sequenced using the forward primer while the nuclear fragments were sequenced for both strands.

V.II.b. Molecular analyses

Sequences were checked and edited in the software CodonCodeAligner 3.7.1 (Codon Code Corporation, Dedham, MA, USA). All sequences were submitted to Genbank (accession numbers ####-#### (to be added upon manuscript acceptance). The alignment was performed using the Clustaw algorithm as implemented in the software MEGA5.0 (Tamura et al., 2011). Because of the presence of several gaps in the 16S sequences we performed an alignment comparison with the program SOAP (Löytynoja and Milinkovitch, 2001) and the unstable regions were excluded of the phylogenetic analysis. To estimate the best fit model of substitution we used Akaike Information Criteria (AIC, Akaike, 1974) implemented in jModel Test 0.1 (Posada, 2008). A gene tree based on

the 16S sequences was estimated using Bayesian Inference with Markov Chain Monte Carlo (MCMC) algorithm in the software MrBayes 3.1.2.(Akaike, 1974). To test the monophyly of the complex and to check for possible misidentification we used as outgroup several species closely related to *I. guentheri* (*I. nasuta*, *I. izecksoni*, *I. oea*, *I. erythromera*, *I. venancioi*). We ran two chains with different heating settings, for 20 x 10^6 iterations, sampling every 1000. We checked for chain mixing, effective sample size (ESS) and convergence between runs using the program Tracer 1.5 (Rambaut and Drummond, 2009). The first 4000 trees were discarded as burn-in. Based on the generated topology we calculated uncorrected pairwise *p*-distances between major lineages and the mean *p*-distances within lineages using the entire sequenced fragment with "complete deletion" in MEGA5. The RAG1 sequences were phased using PHASE algorithm (Stephens and Scheet, 2005; Stephens et al., 2001) through the program DNAsp5 (Librado and Rozas, 2009). All estimated allele pairs with a probability lower than 0.90 were excluded of the analysis and the remaining haplotypes used to construct a Median-Joining haplotype network using NETWORK 4.6 (www.fluxus-engineering.com). Nuclear haplotypes were labeled according to the major mitochondrial lineages. To exclude that the allele pairs with low probability values from PHASE could show haplotype sharing among major mitochondrial lineages we also constructed a haplotype network with the complete list of alleles generated by PHASE (data not shown). Additionally we calculated the genetic diversity of the RAG1 sequences and we performed an AMOVA in ARLEQUIN 3.1 (Excoffier et al., 2005), to assess the degree of variation in the nuclear gene among localities and mitochondrial groups.

V.II.c. Bioacoustic analysis

We analyzed 56 advertisement calls of 16 specimens of the *I. guentheri* complex, recorded from seven localities: Monte Verde, municipality of Camanducaia, state of Minas Gerais; Santa Virgínia, municipality of São Luiz do Paraitinga, state of São Paulo; Três Picos State Park, municipality of Nova Friburgo (two different call patterns); Morumbeca, Desengano State Park, municipality of Santa Maria Madalena; Tijuca National Park, municipality of Rio de Janeiro; both in the state of Rio de Janeiro; and Nova Rússia, municipality of Blumenau; Pousada das Hortênsias, municipality of São Bonifácio; both in the state of Santa Catarina. Samples of the same specimens were also included in the molecular assessment. Calls were recorded in the field with a Marantz Professional PMD660 digital recorder coupled to an external Sennheiser directional microphone, or with a TASCAM DR-07 digital recorder with internal microphone. Additional calls were obtained from the Célio F.B. Haddad sound collection (CFBH). We performed sound analyses and obtained waveforms and spectrograms with Raven 1.2.1 software (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY, USA). Advertisement calls of the *I. guentheri* complex are series of notes with regular internote intervals, and especially differing in note repetition rate among lineages (see below). Measured

bioacoustic variables include: call duration, call dominant frequency, number of notes per call, note repetition rate (number of notes – 1 / call duration), frequency modulation (comparing first to last note dominant frequencies), and call amplitude modulation. The repetition rate in the first and last five notes were respectively assessed to calculate the percentage of acceleration in the note repetition rate during the call.

V.III. Results

V.III.a. Molecular differentiation

The alignment of the 16S sequences resulted in 488 bp including highly variable regions, and 320 bp excluding these. The estimated best fit model was JC+Gamma. The 16S tree recovers the monophyly of the *I. guentheri* complex which contains 6 well defined mitochondrial lineages with high node support (**Figure 6**). The inter-lineage and intra-lineage *p*-distances are not overlapping (**Table 1and Table 2**). All six lineages are distributed as a mosaic throughout the Atlantic Forest. In some localities two mitochondrial lineages occur sympatrically. Samples from the type locality of *I. guentheri* (Tijuca National Park) constitute a unique mitochondrial lineage and its sister group is found at the center and north of Rio de Janeiro state (Três Picos State Park and Desengano State Park) (**Figure 7**). Therefore, based on the congruence of mitochondrial and nuclear results which are corroborated by bioacoustics (see below), the Tijuca National Park lineage is referred to as being the true *I. guentheri* whereas the other lineages are assigned to *I. henselii* and to four candidates to new species (CS) named CS1 to CS4.

The most basal lineage (CS 4) is distributed in the northeastern portion of São Paulo (SP) state, in the border region with Minas Gerais (MG) and Rio de Janeiro (RJ) states, at the massifs of *Serra da Mantiqueira* and *Serra do Mar*. Apart from the two lineages only found in RJ, all the other lineages are present in this geographical area. The two crown lineages are widely distributed towards the south. Of these, the lineage with the southernmost distribution is here considered to represent *I. henselii*, given that it contains samples a priori assigned to that species. If this entire lineage truly is *I. henselii*, then this species is distributed further north than previously described (see Kwet and Solé 2005).

The RAG1 haplotype network revealed a high haplotype diversity (0.95). Apart from one central haplotype, which is shared among three mitochondrial lineages (CS 4, 2 and *I. guentheri*), there is no nuclear haplotype sharing among mitochondrial lineages (**Figure 7**) whereas within each lineage some haplotypes are shared among samples from different localities (**Supplementary Table**

1). According to AMOVA analysis most of the variation in the nuclear gene occurs between localities (**Table 2**).

V.III.b. Bioacoustics

All the analyzed advertisement calls exhibits great amplitude modulation, with an increasing intensity towards the end of the call. Dominant frequency does not vary much among lineages. Calls are frequency-modulated with a weakly ascendant frequency. Notes within and between mitochondrial lineages show very similar structure and duration. In contrast, other variables exhibit great differences among the lineages. Two variables in particular distinguish the call recorded at the type locality of *I. guentheri* from that of other lineages. Specimens of *Ischnocnema guentheri* from the type locality have the longest call duration (26.3-41.9 s) while *I. henselii* has intermediate values (14.6-15.9 s) and all other lineages have short calls (0.8-9.5 s; combined values). Calls of *I. guentheri* from the type locality are also different in their lower note repetition rate (2.2-3.5 notes/s). This dramatically differs from CS 2, its sister group (20.9-30.7 notes/s), and also from the other lineages (6.6-19.7 notes/s; combined values). Despite some overlap, *I. guentheri* plus *I. henselii* and CS 2 present higher numbers of notes per call (57-199 notes per call, x=111.8; combined values) than the other lineages (15-72 notes per call, x=37.3; combined values). Note repetition rate accelerates during the calls of *I. guentheri* (31-121% of acceleration) similar to *I. henselii* (106-125% of acceleration), CS 2 (18-186% of acceleration), and CS 3 (8% of deceleration to 62% of acceleration), but differing from CS 1 (no changes in the repetition rate) and CS 4 (23% of deceleration to 2% of acceleration).

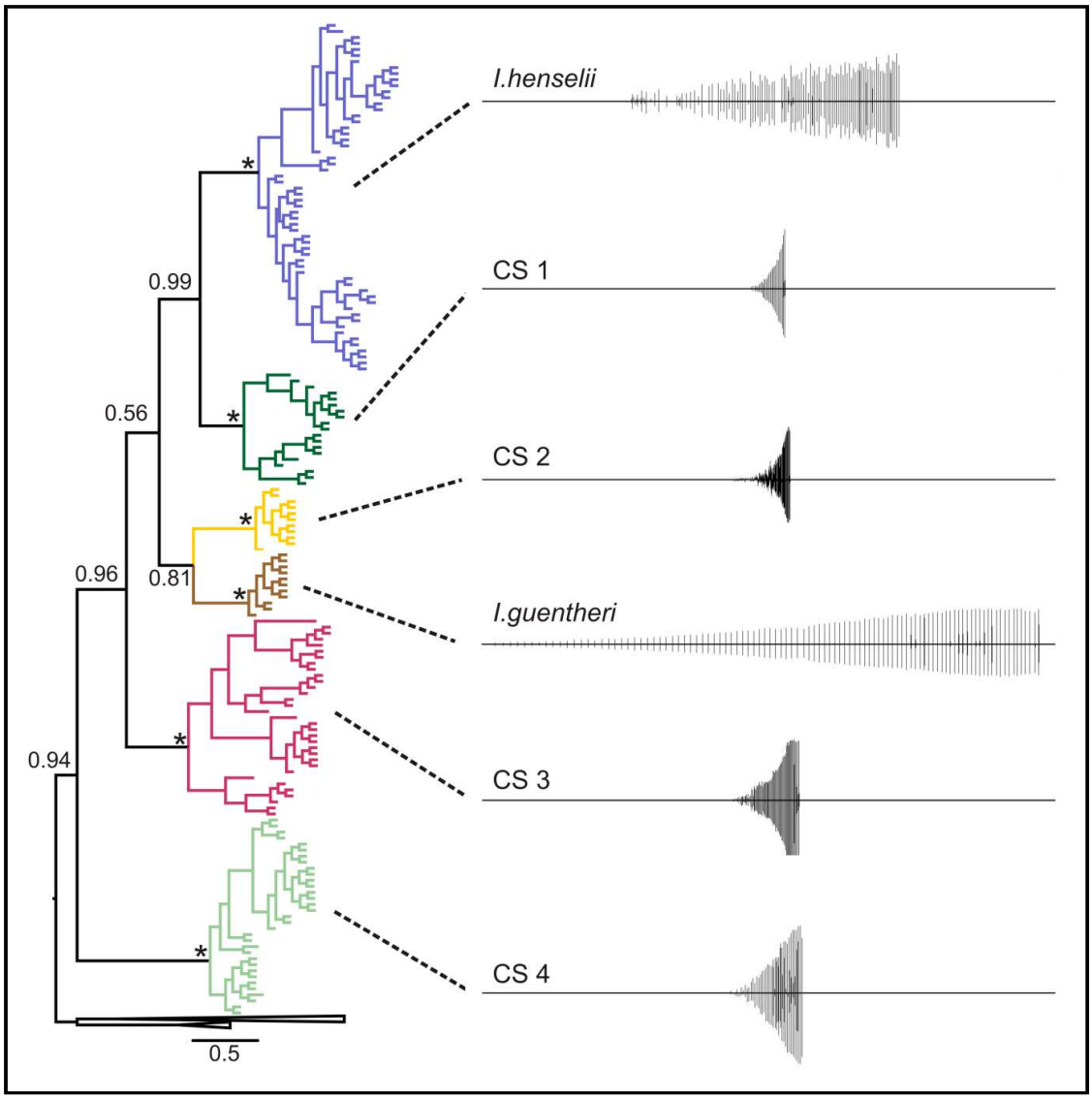

Figure 6*:* 50%-majority rule consensus tree derived from Bayesian inference analysis of partial 16S sequences, showing the six main mitochondrial lineages with respective call oscilograms of *Ischnocnema guentheri* complex. All oscillograms have the total length of 32 seconds and show one call (consisting of a series of notes). Node probabilities of 1.0 are shown with an asterisk. The abbreviation CS is used for candidate species hypothesized on the basis of molecular and bioacoustic data. See text and Figure 7 for additional information.

Table 1: Average uncorrected p-distances within lineages and p-distances between lineages.

	Average *p*-distance within lineage	Pairwise *p*-distance				
		I.henselii	CS 1	CS 2	*I.guentheri*	CS 3
I.henselii	0.02					
CS 1	0.02	0.06				
CS 2	0	0.07	0.08			
I.guentheri	0.002	0.08	0.07	0.06		
CS 3	0.04	0.10	0.11	0.09	0.09	
CS 4	0.02	0.12	0.12	0.13	0.12	0.13

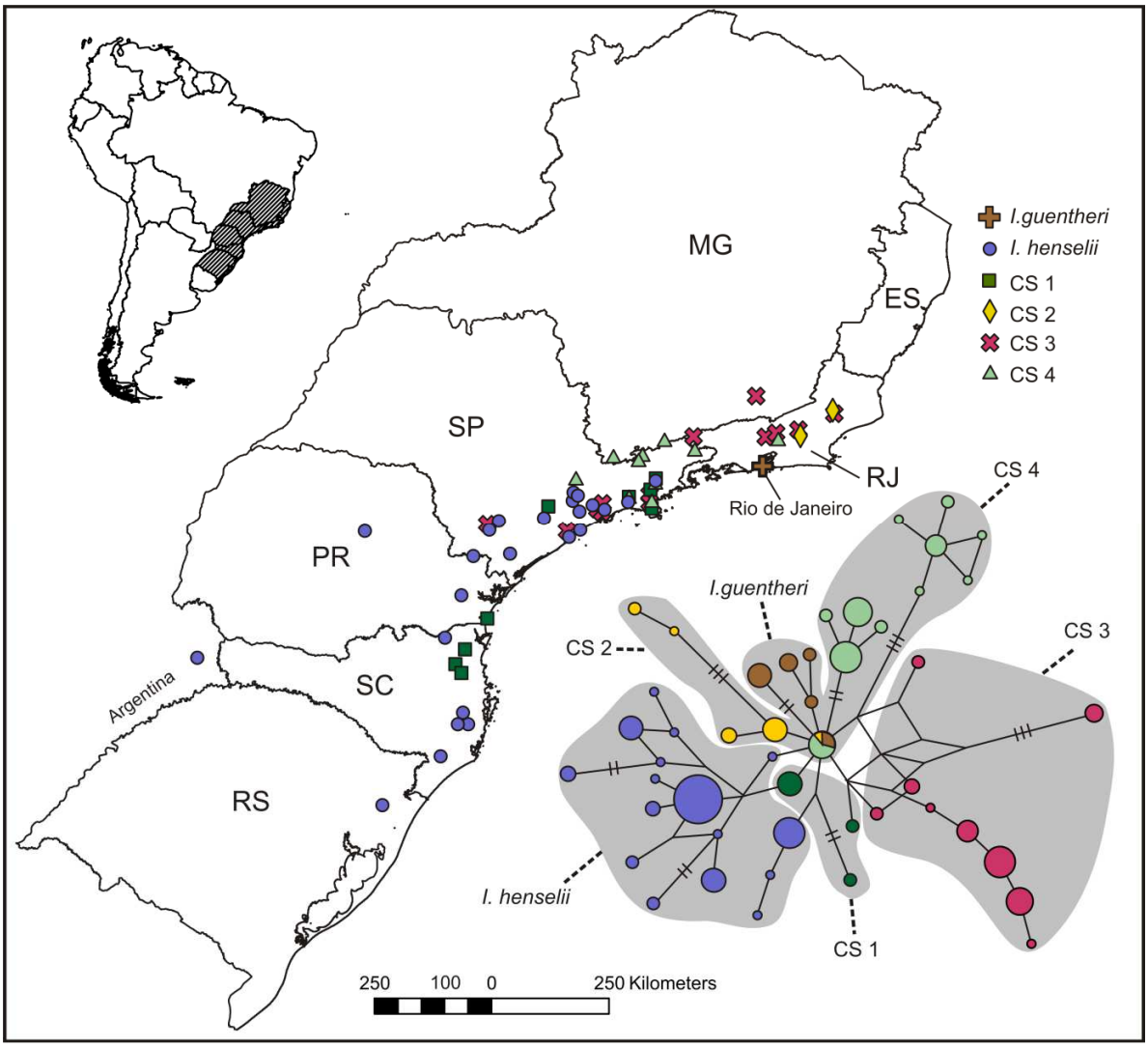

Figure 7: Map showing the distribution of the *Ischnocnema guentheri* complex in the Atlantic forest of Brazil, and nuclear RAG1 haplotype network. Branch lengths are proportional to the number of mutational steps which are shown only in branches with more than one mutational step. Colors of haplotypes in the network are assigned based on the grouping of samples in major mitochondrial clades (Figure 6).

Table 2: AMOVA analysis of the RAG1 sequences. As structure setting each locality was set as a different population and each species as a different group.

Source of variation	Percentage of variation
Among Groups	35.09
Among Population within groups	41.11
Within Populations	23.80

V.IV. Discussion

V.IV.a. Species assessment and overall phylogeographycal pattern

Our study combines a molecular assessment of variation with a preliminary analysis of advertisement calls in the *I. guentheri* complex. As other studies have found in other Neotropical anurans (Fouquet et al., 2007a; Funk et al., 2012), our results support this putatively widespread species being a complex of numerous well-differentiated and partly syntopic species. Two of these species can be readily associated to *I. guentheri*, *I. henselii*, whereas the other four are considered here as candidates to new species (CS 1-4).

Call differences mainly related to temporal structure as observed here are consistent with species level differentiation, as reported by Heyer (1984) for other related species of frogs. The most conclusive evidence for species-level differentiation comes from syntopic occurrence without admixture. For instance, from the municipality of Teresópolis we sequenced six specimens of CS 3 and four specimens of CS 4, and found no haplotype sharing in RAG1 between specimens previously assigned to either CS based on their mitochondrial sequence. The same was found in the locality of São Luiz do Paraitinga where 22 specimens of CS 4 and two specimens of CS 1 were sequenced for the two markers. Another evidence is the presence of individuals displaying different advertisement calls at the locality of Três Picos State Park which were further assigned to different mitochondrial lineages (CS 2 and CS 3).

In addition to the strong evidence that support these six lineages as valid species, the 16S divergences and the RAG1 AMOVA result might indicate the existence of even more species within the complex, which would require a further species-level splitting of each mitochondrial lineage. The amount of mitochondrial divergence between populations is high, exclusive nuclear haplotypes are found in some localities (**Supplementary Table 1**) and the RAG1 haplotype network is not fully congruent with the mitochondrial tree (**Figure 6and Figure 7**). While these results on one hand suggest a low vagility of species within this complex, the wide distribution of most of the lineages indicate the contrary. Although this scenario seems entangled, it agrees with the biology of the species, its reproductive mode and past forest fragmentation/connection. As most of the species of direct developing frogs are restricted to forest areas, their dispersion capacity is tightly linked to forest continuity. On the other hand, within continuous forests they can move freely and disperse more easily, because they do not depend on water bodies for breeding. In such case, forest fragmentation will cause isolation and diversification, while forest connections allow easy dispersion and contact between diversified lineages. Streicher et al. (2009) combined this idea with topography to propose a model explaining a similar pattern found in another direct developing frog group from high lands Central America, the *Craugastor ponticiferus* complex. Based on a classical model of mountain

refugia, they consider mountains as playing an important role in the diversification and distribution of the species. When suitable habitat is restricted to high lands, mountains act as islands and diversification occurs, whereas in periods when suitable habitat occurs in lowlands, divergent lineages get into contact. Thus, successive climatic oscillations can generate the pattern found, with different lineages co-occurring at some places. This model may also apply to *I. guentheri*. The species occurs on slopes and in highlands and there is some evidence that it is more abundant at higher altitudes (Giaretta et al., 1997). Studies in other tropical regions, such as the Australian Wet Tropics and Madagascar, have already provided evidence for mountains being key regions for speciation in at least some groups of frogs (Graham et al., 2006; Wollenberg et al., 2008).

We also find that the distribution of *I. guentheri*, with four lineages present in the region of São Paulo state, partially agrees with paleo-models of the Atlantic Forest and related anurans for the Pleistocene (Carnaval et al., 2009; Carnaval and Moritz, 2008; Thomé et al., 2010), but the presence of endemic lineages in RJ suggests the existence of micro-refuge and other stable areas, at least for this species complex. Species distribution reported for the genus *Brachycephalus* also supports this hypothesis (Clemente-Carvalho et al., 2011). However, as proposed by Thomé et al. (2010), the phylogeographical pattern for Atlantic Forest species might be the result of a complex scenario with a combination of different factors. These authors concluded that paleoecological distribution models support a scenario of habitat fragmentation associated with glacial cycling, but observed limited congruence of phylogeographical patterns with the refugia. They found that some genetic breaks geographically coincide with putative barriers associated to neotectonic activity, but finer-scale sampling will be necessary to test the relative importance of distinct isolation mechanisms.

Because of their necessity to forested areas and humidity for reproduction, and their non dependence to water bodies, the *Ischnocnema guentheri* complex and other co-distributed direct developing frogs are a suitable group for testing paleoclimatic fragmentation models of the Brazilian Atlantic Forest. Further phylogeographic analysis of these amphibians will help in the understanding of diversification pattern of terraranan frogs and other anurans in the Atlantic Forest region.

V.IV.b. Current state of the Ischnocnema guentheri complex

Ischnocnema henselii, previously restricted to Santa Catarina (SC) state and north of Rio Grande do Sul (RS) state, was found to be distributed further north, up to northeast of São Paulo (SP) state. The study by Kwet and Solé (2005) that led to the resurrection of *I. henselii* was based on the comparison of calls from north of RS, SC, from two localities of SP (Serra da Bocaina and Boracéia) in Brazil, and from Misiones, Argentina. The authors found *I. henselii* calls in RS and SC, but apparently because of their small sample size they could not detect the species in Paraná (PR) and SP. Furthermore, because

of the proximity of Serra da Bocaina, SP, with the type locality of *I. guentheri*, they suggested that the calls from that locality corresponded to *I. guentheri* sensu stricto. The animals we analyzed from Serra da Bocaina, in contrast, belong to two other, distinct lineages (CS 3 and CS 4). The call parameters we found for the CS 4 are similar to the parameters described by Kwet and Solé (2005) for this locality, especially note repetition rate and call duration. In addition, a previous call description of *I. guentheri* from Pirabeiraba, SC (Heyer 1984:1990) agrees with calls from northern SC (Kwet and Solé 2005) and they all present call parameters similar to what found for CS 1, which means that a formal redescription of *I. guentheri* sensu stricto still is required.

The type locality of *Ischnocnema guentheri* is the ”Rio dos Macacos”, Rio de Janeiro (Häupl et al., 1994), a stream that crosses the Tijuca Forest and the Botanical Garden of Rio de Janeiro (Lucas and Cunha, 2007). According to our results *I. guentheri* sensu stricto is endemic to the Tijuca Forest and thus to the area surrounding its type locality, one of the largest urban forests of the world located in the middle of Rio de Janeiro city (Freitas et al., 2006). As we have not analyzed other regions of the city, we cannot discard the possibility that the species is also present in adjacent forested areas (e.g. the large Pedra Branca State Park). However, its apparent absence from other surveyed localities at a wider distance from Rio de Janeiro city leads us to hypothesize that this species might indeed be microendemic to a very small geographical area which largely corresponds to the almost completely urbanized area of Rio de Janeiro city. In a recent work, Newman et al. (2012) called for attention on the importance of urban areas for conservation after their surprising discovery of an undescribed species of leopard frog in a densely urbanized area in the surroundings of New York City. The importance of urban areas for conservation of biodiversity is also highlighted by the case of *I. guentheri*, and calls for an integration of conservation planning and landuse planning in such landscapes (e.g., Gordon et al., 2009). Our study reinforces the importance of this reserve and adjacent forested areas for the conservation of the biodiversity of the Atlantic Forest. Molecular studies of Tijuca Forest fauna are extremely rare, and we suspect that other cryptic species might be also endemic to the city of Rio de Janeiro.

The Red List status of *I. guentheri* currently is of Least Concern (IUCN 2011). Despite the restricted distribution of the species, and its occurrence in a highly urbanized area we do not propose at this time a change of its IUCN threat status. The Floresta da Tijuca National Park is a protected area without obvious threats to its surface, and we found a high number of specimens during field work in 2010. Nevertheless, we recommend that the status of this species needs to be carefully re-evaluated in the framework of the next update of the Global Amphibian Assessment, given its possibly very small extent of occurrence in Tijuca Park, a largely secondary forest that has been replanted in the 19th century after its initial degradation, and that is used by the Rio de Janeiro population for recreation and tourism. *Ischnocnema henselii* is currently listed as of Least Concern (IUCN 2011), and our data

confirm this assessment, by even further expanding the known range of this widespread species. In general, even in the case of lineages with restricted distribution, the area of occurrence of each of them typically encompasses one or several protected areas, especially at high elevations. Moreover, direct developing frog community might suffer less impact caused by habitat split (Becker et al., 2007). Therefore we do not suggest any specific urgent conservation measure. However, a taxonomic revision and more detailed range assessment of each of the yet undescribed species is necessary. Especially if such future studies would result in the necessity of a partition of the species into even more fine-scale units than those proposed here, then some of these units might be characterized by restricted ranges in areas of ongoing habitat degradation and thus might qualify for an inclusion in one of the IUCN threat categories.

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Supplementary Table 1: Haplotypes identified in the RAG1 analysis as in the respective network (Figure 7) with respective sample numbers, voucher specimens, localities and species / candidate species assignment. Non available voucher specimens are specified as *na*

Haplotype	Tissue number	Voucher specimen	Locality	State	Species
Hap 1	CFBH-T 10987	CFBH 23298	Tapiraí	SP	*I. henselii*
Hap 1	CFBH-T 10987	CFBH 23298	Tapiraí	SP	*I. henselii*
Hap 2	CFBH-T 13127	CFBH 27471	Adrianópolis	SC	*I. henselii*
Hap 3	CFBH-T 13127	CFBH 27471	Adrianópolis	SC	*I. henselii*
Hap 3	CFBH-T 13826	*na*	Misiones	ARGENTINA	*I. henselii*
Hap 3	CFBH-T 13826	*na*	Misiones	ARGENTINA	*I. henselii*
Hap 3	MCP 10561	MCP 10561	Campo Belo do Sul	SC	*I. henselii*
Hap 3	MCP 10561	MCP 10561	Campo Belo do Sul	SC	*I. henselii*
Hap 3	MCP 10565	MCP 10565	Campo Belo do Sul	SC	*I. henselii*
Hap 3	MCP 10565	MCP 10565	Campo Belo do Sul	SC	*I. henselii*
Hap 4	CFBH-T 13207	CFBH 27551	São Bonifacio	SC	*I. henselii*
Hap 4	MCP 10703	MCP 10703	São Francisco de Paula	RS	*I. henselii*
Hap 4	MCP 10703	MCP 10703	São Francisco de Paula	RS	*I. henselii*
Hap 5	CFBH-T 13207	CFBH 27551	São Bonifacio	SC	*I. henselii*
Hap 6	CFBH-T 13208	CFBH 27552	São Bonifacio	SC	*I. henselii*
Hap 6	CFBH-T 13208	CFBH 27552	São Bonifacio	SC	*I. henselii*
Hap 6	CFBH-T 13209	CFBH 27553	São Bonifacio	SC	*I. henselii*
Hap 6	CFBH-T 13209	CFBH 27553	São Bonifacio	SC	*I. henselii*
Hap 6	CFBH-T 1924	CFBH 8497	Treviso	SC	*I. henselii*
Hap 6	CFBH-T 1924	CFBH 8497	Treviso	SC	*I. henselii*
Hap 6	CFBH-T 2088	CFBH 9367	Anitápolis	SC	*I. henselii*
Hap 6	CFBH-T 2088	CFBH 9367	Anitápolis	SC	*I. henselii*
Hap 6	CFBH-T 2891	UFSC 00934	Anitápolis	SC	*I. henselii*
Hap 6	MCP 10702	MCP 10702	São Francisco de Paula	RS	*I. henselii*
Hap 6	MCP 10704	MCP 10704	São Francisco de Paula	RS	*I. henselii*
Hap 6	MCP 10704	MCP 10704	São Francisco de Paula	RS	*I. henselii*
Hap 6	MCP 10762	MCP 10762	São Francisco de Paula	RS	*I. henselii*
Hap 7	CFBH-T 13210	CFBH 27554	São Bonifacio	SC	*I. henselii*
Hap 7	CFBH-T 13210	CFBH 27554	São Bonifacio	SC	*I. henselii*
Hap 7	CFBH-T 2089	CFBH 9368	Anitápolis	SC	*I. henselii*
Hap 7	CFBH-T 2089	CFBH 9368	Anitápolis	SC	*I. henselii*
Hap 7	CFBH-T 2891	UFSC 00934	Anitápolis	SC	*I. henselii*
Hap 7	MCP 10702	MCP 10702	São Francisco de Paula	RS	*I. henselii*
Hap 7	MCP 10762	MCP 10762	São Francisco de Paula	RS	*I. henselii*
Hap 8	CFBH-T 1915	CFBH 9138	São Paulo	SP	*I. henselii*
Hap 8	CFBH-T 1915	CFBH 9138	São Paulo	SP	*I. henselii*
Hap 8	CFBH-T 3109	CFBH 11655	Cotia	SP	*I. henselii*
Hap 8	CFBH-T 3146	*na*	Itanhaém	SP	*I. henselii*
Hap 8	CFBH-T 3146	*na*	Itanhaém	SP	*I. henselii*
Hap 8	CFBH-T 3147	*na*	Itanhaém	SP	*I. henselii*

Hap 8	CFBH-T 3153	*na*	Itanhaém	SP	*I. henselii*
Hap 8	CFBH-T 3153	*na*	Itanhaém	SP	*I. henselii*
Hap 8	CFBH-T 3156	*na*	São Bernardo do Campo	SP	*I. henselii*
Hap 8	CFBH-T 3162	*na*	Itanhaém	SP	*I. henselii*
Hap 8	CFBH-T 3162	*na*	Itanhaém	SP	*I. henselii*
Hap 8	CFBH-T 3167	*na*	Biritiba Mirin	SP	*I. henselii*
Hap 8	CFBH-T 3167	*na*	Biritiba Mirin	SP	*I. henselii*
Hap 8	CFBH-T 3871	CFBH 12214	Itanhaém	SP	*I. henselii*
Hap 8	CFBH-T 3871	CFBH 12214	Itanhaém	SP	*I. henselii*
Hap 8	CFBH-T 3880	*na*	Itanhaém	SP	*I. henselii*
Hap 8	CFBH-T 3880	*na*	Itanhaém	SP	*I. henselii*
Hap 8	CFBH-T 3884	CFBH 12299	São Bernardo do Campo	SP	*I. henselii*
Hap 8	CFBH-T 3886	*na*	São Bernardo do Campo	SP	*I. henselii*
Hap 8	CFBH-T 3886	*na*	São Bernardo do Campo	SP	*I. henselii*
Hap 8	CFBH-T 3900	CFBH 12301	São Bernardo do Campo	SP	*I. henselii*
Hap 8	CFBH-T 3900	CFBH 12301	São Bernardo do Campo	SP	*I. henselii*
Hap 8	CFBH-T 3901	*na*	São Bernardo do Campo	SP	*I. henselii*
Hap 8	CFBH-T 3901	*na*	São Bernardo do Campo	SP	*I. henselii*
Hap 8	CFBH-T 3909	CFBH 12243	São Bernardo do Campo	SP	*I. henselii*
Hap 8	CFBH-T 3909	CFBH 12243	São Bernardo do Campo	SP	*I. henselii*
Hap 8	CFBH-T 3917	*na*	Biritiba Mirin	SP	*I. henselii*
Hap 8	CFBH-T 3917	*na*	Biritiba Mirin	SP	*I. henselii*
Hap 8	CFBH-T 6921	CFBH 17651	Itanhaém	SP	*I. henselii*
Hap 9	CFBH-T 3063	CFBH 11039	Piraquara	SP	*I. henselii*
Hap 10	CFBH-T 3063	CFBH 11039	Piraquara	SP	*I. henselii*
Hap 11	CFBH-T 3064	CFBH 11040	Piraquara	SP	*I. henselii*
Hap 12	CFBH-T 3064	CFBH 11040	Piraquara	SP	*I. henselii*
Hap 13	CFBH-T 3109	CFBH 11655	Cotia	SP	*I. henselii*
Hap 14	CFBH-T 3147	*na*	Itanhaém	SP	*I. henselii*
Hap 14	CFBH-T 3884	CFBH 12299	São Bernardo do Campo	SP	*I. henselii*
Hap 14	CFBH-T 6921	CFBH 17651	Itanhaém	SP	*I. henselii*
Hap 15	CFBH-T 3156	*na*	São Bernardo do Campo	SP	*I. henselii*
Hap 16	CFBH-T 8755	CFBH 13527	Peruibe	SP	*I. henselii*
Hap 16	CFBH-T 8755	CFBH 13527	Peruibe	SP	*I. henselii*
Hap 17	CFBH-T 13152	CFBH 27496	Pomerode	SC	CS 1
Hap 17	CFBH-T 13152	CFBH 27496	Pomerode	SC	CS 1
Hap 17	CFBH-T 13178	CFBH 27522	Blumenau	SC	CS 1
Hap 17	CFBH-T 13178	CFBH 27522	Blumenau	SC	CS 1
Hap 17	CFBH-T 13181	CFBH 27525	Blumenau	SC	CS 1
Hap 17	CFBH-T 13181	CFBH 27525	Blumenau	SC	CS 1
Hap 17	MCP 8167	MCP 8167	Águas Mornas	SC	CS 1
Hap 17	MCP 8167	MCP 8167	Águas Mornas	SC	CS 1
Hap 18	CFBH-T 6000	CFBH 15035	Ilha Bela	SP	CS 1
Hap 18	CFBH-T 6000	CFBH 15035	Ilha Bela	SP	CS 1
Hap 19	CFBH-T 6549	*na*	São Luiz do Paraitinga	SP	CS 1

Hap 19	CFBH-T 6549	*na*	São Luiz do Paraitinga	SP	CS 1
Hap 20	CFBH-T 12972	CFBH 27318	Parque estadual do Desengano	RJ	CS 2
Hap 20	CFBH-T 12974	CFBH 27320	Parque estadual do Desengano	RJ	CS 2
Hap 20	CFBH-T 13018	CFBH 27359	Parque estadual do Desengano	RJ	CS 2
Hap 20	CFBH-T 12899	CFBH 26991	Floresta Tijuca	RJ	*I. guentheri*
Hap 20	CFBH-T 2300	CFBH 9892	Campos do Jordão	SP	CS 4
Hap 20	CFBH-T 2308	CFBH 9917	Campos do Jordão	SP	CS 4
Hap 20	CFBH-T 2308	CFBH 9917	Campos do Jordão	SP	CS 4
Hap 20	CFBH-T 2312	*na*	Campos do Jordão	SP	CS 4
Hap 20	CFBH-T 652	CFBH 6724	Camanducaia	SP	CS 4
Hap 20	CFBH-T 652	CFBH 6724	Camanducaia	SP	CS 4
Hap 21	CFBH-T 12972	CFBH 27318	Parque estadual do Desengano	RJ	CS 2
Hap 21	CFBH-T 12975	CFBH 27321	Parque estadual do Desengano	RJ	CS 2
Hap 21	CFBH-T 12976	CFBH 27322	Parque estadual do Desengano	RJ	CS 2
Hap 21	CFBH-T 13018	CFBH 27359	Parque estadual do Desengano	RJ	CS 2
Hap 22	CFBH-T 12973	CFBH 27319	Parque estadual do Desengano	RJ	CS 2
Hap 22	CFBH-T 13016	CFBH 27357	Parque estadual do Desengano	RJ	CS 2
Hap 23	CFBH-T 12973	CFBH 27319	Parque estadual do Desengano	RJ	CS 2
Hap 23	CFBH-T 12974	CFBH 27320	Parque estadual do Desengano	RJ	CS 2
Hap 23	CFBH-T 12975	CFBH 27321	Parque estadual do Desengano	RJ	CS 2
Hap 23	CFBH-T 12976	CFBH 27322	Parque estadual do Desengano	RJ	CS 2
Hap 23	CFBH-T 12978	CFBH 27324	Parque estadual do Desengano	RJ	CS 2
Hap 23	CFBH-T 12978	CFBH 27324	Parque estadual do Desengano	RJ	CS 2
Hap 23	CFBH-T 13015	CFBH 27356	Parque estadual do Desengano	RJ	CS 2
Hap 24	CFBH-T 13015	CFBH 27356	Parque estadual do Desengano	RJ	CS 2
Hap 24	CFBH-T 13016	CFBH 27357	Parque estadual do Desengano	RJ	CS 2
Hap 25	CFBH-T 12896	CFBH 26988	Floresta Tijuca	RJ	*I. guentheri*
Hap 25	CFBH-T 12898	CFBH 26990	Floresta Tijuca	RJ	*I. guentheri*
Hap 25	CFBH-T 12899	CFBH 26991	Floresta Tijuca	RJ	*I. guentheri*
Hap 25	CFBH-T 12901	CFBH 26993	Floresta Tijuca	RJ	*I. guentheri*
Hap 25	CFBH-T 12901	CFBH 26993	Floresta Tijuca	RJ	*I. guentheri*
Hap 25	CFBH-T 12902	CFBH 26994	Floresta Tijuca	RJ	*I. guentheri*
Hap 25	CFBH-T 13102	CFBH 27443	Floresta Tijuca	RJ	*I. guentheri*
Hap 26	CFBH-T 12896	CFBH 26988	Floresta Tijuca	RJ	*I. guentheri*
Hap 26	CFBH-T 12902	CFBH 26994	Floresta Tijuca	RJ	*I. guentheri*
Hap 26	CFBH-T 13102	CFBH 27443	Floresta Tijuca	RJ	*I. guentheri*
Hap 27	CFBH-T 12898	CFBH 26990	Floresta Tijuca	RJ	*I. guentheri*
Hap 28	CFBH-T 12900	CFBH 26992	Floresta Tijuca	RJ	*I. guentheri*
Hap 28	CFBH-T 12900	CFBH 26992	Floresta Tijuca	RJ	*I. guentheri*
Hap 29	CFBH-T 10780	*na*	Itatiaia	RJ	CS 3
Hap 29	CFBH-T 4370	CFBH 13931	Petrópolis	RJ	CS 3
Hap 30	CFBH-T 10780	*na*	Itatiaia	RJ	CS 3
Hap 31	CFBH-T 12348	CFBH 24772	Teresópolis	RJ	CS 3
Hap 31	CFBH-T 12351	CFBH 24773	Teresópolis	RJ	CS 3
Hap 31	CFBH-T 12351	CFBH 24773	Teresópolis	RJ	CS 3

Hap 31	CFBH-T 12353	CFBH 24774	Teresópolis	RJ	CS 3
Hap 32	CFBH-T 12348	CFBH 24772	Teresópolis	RJ	CS 3
Hap 32	CFBH-T 12353	CFBH 24774	Teresópolis	RJ	CS 3
Hap 33	CFBH-T 12970	*na*	Juiz de Fora	MG	CS 3
Hap 33	CFBH-T 12970	*na*	Juiz de Fora	MG	CS 3
Hap 33	CFBH-T 4370	CFBH 13931	Petrópolis	RJ	CS 3
Hap 34	CFBH-T 13066	CFBH 27407	Parque Estadual dos Três Picos	RJ	CS 3
Hap 34	CFBH-T 13066	CFBH 27407	Parque Estadual dos Três Picos	RJ	CS 3
Hap 34	CFBH-T 13087	CFBH 27428	Parque Estadual dos Três Picos	RJ	CS 3
Hap 34	CFBH-T 13087	CFBH 27428	Parque Estadual dos Três Picos	RJ	CS 3
Hap 34	CFBH-T 13089	CFBH 27430	Parque Estadual dos Três Picos	RJ	CS 3
Hap 34	CFBH-T 13089	CFBH 27430	Parque Estadual dos Três Picos	RJ	CS 3
Hap 35	CFBH-T 1960	CFBH 9230	Cubatão	SP	CS 3
Hap 35	CFBH-T 1960	CFBH 9230	Cubatão	SP	CS 3
Hap 35	CFBH-T 1961	CFBH 9236	Cubatão	SP	CS 3
Hap 35	CFBH-T 3949	*na*	Santos	SP	CS 3
Hap 35	CFBH-T 3960	CFBH 12252	Peruíbe	SP	CS 3
Hap 35	CFBH-T 4323	CFBH 11350	Cubatão	SP	CS 3
Hap 35	CFBH-T 4323	CFBH 11350	Cubatão	SP	CS 3
Hap 35	CFBH-T 5531	CFBH 11554	Cubatão	SP	CS 3
Hap 35	CFBH-T 6085	CFBH 15044	Ilha Bela	SP	CS 3
Hap 36	CFBH-T 1961	CFBH 9236	Cubatão	SP	CS 3
Hap 36	CFBH-T 3807	CFBH 12146	Caraguatatuba	SP	CS 3
Hap 36	CFBH-T 3807	CFBH 12146	Caraguatatuba	SP	CS 3
Hap 36	CFBH-T 3949	*na*	Santos	SP	CS 3
Hap 36	CFBH-T 5531	CFBH 11554	Cubatão	SP	CS 3
Hap 36	CFBH-T 6022	CFBH 15036	Ilha Bela	SP	CS 3
Hap 36	CFBH-T 6022	CFBH 15036	Ilha Bela	SP	CS 3
Hap 36	CFBH-T 6085	CFBH 15044	Ilha Bela	SP	CS 3
Hap 36	CFBH-T 6102	CFBH 15038	Ilha Bela	SP	CS 3
Hap 36	CFBH-T 6102	CFBH 15038	Ilha Bela	SP	CS 3
Hap 36	CFBH-T 6106	CFBH 15045	Ilha Bela	SP	CS 3
Hap 36	CFBH-T 6106	CFBH 15045	Ilha Bela	SP	CS 3
Hap 37	CFBH-T 3960	CFBH 12252	Peruíbe	SP	CS 3
Hap 38	CFBH-T 10368	*na*	Serra da Bocaina	SP	CS 4
Hap 38	CFBH-T 10368	*na*	Serra da Bocaina	SP	CS 4
Hap 39	CFBH-T 11643	CFBH 24143	Campos do Jordão	SP	CS 4
Hap 40	CFBH-T 11643	CFBH 24143	Campos do Jordão	SP	CS 4
Hap 41	CFBH-T 11651	CFBH 24141	Marmelópolis	MG	CS 4
Hap 41	CFBH-T 11651	CFBH 24141	Marmelópolis	MG	CS 4
Hap 42	CFBH-T 12357	CFBH 24769	Teresópolis	RJ	CS 4
Hap 42	CFBH-T 12357	CFBH 24769	Teresópolis	RJ	CS 4
Hap 42	CFBH-T 12361	CFBH 24770	Teresópolis	RJ	CS 4
Hap 42	CFBH-T 12361	CFBH 24770	Teresópolis	RJ	CS 4
Hap 42	CFBH-T 483	CFBH 6493	São Luiz do Paraitinga	SP	CS 4

Hap 42	CFBH-T 483	CFBH 6493	São Luiz do Paraitinga	SP	CS 4
Hap 42	CFBH-T 6561	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 42	CFBH-T 6561	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 42	CFBH-T 6563	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 42	CFBH-T 6566	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 42	CFBH-T 6568	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 42	CFBH-T 6570	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 42	CFBH-T 6571	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 43	CFBH-T 2300	CFBH 9892	Campos do Jordão	SP	CS 4
Hap 44	CFBH-T 2312	*na*	Campos do Jordão	SP	CS 4
Hap 44	CFBH-T 2313	CFBH 9891	Campos do Jordão	SP	CS 4
Hap 44	CFBH-T 2313	CFBH 9891	Campos do Jordão	SP	CS 4
Hap 44	CFBH-T 656	CFBH 6725	Camanducaia	SP	CS 4
Hap 44	CFBH-T 6942	*na*	Campos do Jordão	SP	CS 4
Hap 44	CFBH-T 6942	*na*	Campos do Jordão	SP	CS 4
Hap 46	CFBH-T 656	CFBH 6725	Camanducaia	SP	CS 4
Hap 47	CFBH-T 6562	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 47	CFBH-T 6564	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 47	CFBH-T 6564	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 47	CFBH-T 6565	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 47	CFBH-T 6565	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 47	CFBH-T 6566	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 47	CFBH-T 6568	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 47	CFBH-T 6569	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 47	CFBH-T 6569	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 47	CFBH-T 6570	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 47	CFBH-T 6571	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 48	CFBH-T 6562	*na*	São Luiz do Paraitinga	SP	CS 4
Hap 48	CFBH-T 6563	*na*	São Luiz do Paraitinga	SP	CS 4

VI. Do continentally widespread species of frogs exist in the Neotropics? Molecular analyses indicate that *Dendropsophus minutus* is a lineage-rich and biogeographically complex taxon.

Abstract

Molecular studies published in recent years revealed a high diversity of divergent amphibian lineages within certain taxonomic units. Nevertheless, these studies were mainly conducted within limited geographical regions. Studies comprising the entire distribution of species that are assumed to occupy vast continental areas across political borders are rare because data sampling is handicapped by financial, logistic and political factors. Such is the case of *Dendropsophus minutus,* a putatively continentally distributed South American Hylidae. Although scientists recognized regional differences in morphology or behavior among populations most studies were conducted within limited geographical areas or even at a national level. As a consequence, the species is either regarded to represent truly widespread single species, or considered to represent complexes of multiple species, each with a more limited distribution. However, there is little evidence for both of these views so far and thus the status of populations remains largely unsolved. As most Neotropical amphibian species are distributed within certain biomes, phylogeographical studies are often focused on specific regions whereas the global continental picture is scarcely studied. Thus, besides the taxonomic importance of analyzing such widespread species, the molecular analyses of the nominal *D. minutus* and related species provide a singular opportunity for investigations on the biogeography of South America. To analyze the molecular diversity and the phylogeographical pattern existent throughout the range of the species we made a collaborative effort bringing together 416 tissue samples of *D. minutus* and closely related species that were analyzed for two mitochondrial markers (the DNA barcode fragment – COI, and 16S rDNA). We performed phylogenetic, demographic and newly developed analysis of phylogeographical reconstruction to access the number of mitochondrial lineages, demographical change through time and the geographical origin of *D. minutus* and related species respectively. We found more than 19 largely alopatric deep mitochondrial lineages within *D. minutus,* most of them with high node support. Ten of these lineages form a monophyletic clade representing the *D. minutus* complex. The others lineages would represent related species forming the *D. minutus* group.The phylogegraphical analysis support an Amazonian origin for the *D. minutus* group with a subsequent dispersal to eastern Brazil where the *D. minutus* complex originates. We found signs of recent demographic expansion in three of the ten lineages within the *D. minutus* complex. The phylogeographical pattern of the *D. minutus* complex largely agree with previous molecular studies of the Brazilian Atlantic Forest fauna.

VI.I. Introduction

At a global scale, the application of molecular methods to evolutionary research has tremendously expedited the discovery of genealogical lineages (Bickford et al., 2007). This is particularly true for amphibians (Köhler et al., 2005; Vences and Köhler, 2008) where the existence of many widespread species, allied to the common perception of a low individual vagility (Duellman and Trueb, 1986), compels the idea that these species should house many internal lineages. Molecular studies published in recent years revealed a high diversity of divergent amphibian lineages within certain geographic regions or taxonomic units (e.g., Fouquet et al., 2007a; Funk et al., 2012; Jansen et al., 2011; Vieites et al., 2009). Integrative approaches, combining distinct lines of evidence, were particularly capable to decipher diversity at the species level (e.g. Biju et al., 2011; Funk et al., 2008; Glaw et al., 2010; Padial and De la Riva, 2009). The synergy between the cheaper costs of molecular studies and the fact that there has never been so many researchers interested in amphibian diversity and taxonomy caused the rates of species descriptions to accelerate significantly in the past two decades (Gehring et al., 2011; Köhler et al., 2005). Furthermore, the improved delimitation of species diversity, transforming once widely distributed species into several species, each with a significantly smaller range, in many cases has notable impact on conservation, as the status of certain populations may easily change from 'least concern' to one of the various threat categories defined by IUCN red list criteria (e.g.Maciel and Nunes, 2010).

Many studies were mainly that discovered a high proportion of cryptic diversity in amphibians were manly target at limited geographical regions only (e.g. Funk et al., 2012; Jansen et al., 2011). One practical problem involved with such analyses is data sampling. Data sampling for species that are assumed to occupy vast continental areas across political borders is handicapped by financial, logistic and political factors. With regard to the Neotropics, the nominal species *Rhinella margaritifera* (Bufonidae); *Scinax ruber* and *Trachycephalus typhonius* (Hylidae); and *Leptodactylus fuscus* (Leptodactylidae) are prominent examples for anuran species once considered to occur in almost the entire tropical lowlands of South America (e.g. Camargo et al., 2006; Fouquet et al., 2007b). Although scientists recognized regional differences in morphology or behaviour among populations of the mentioned taxa, most of their work was conducted within limited geographical areas or even at a national level, and in most cases comparative data from other regions are lacking or unavailable. As a consequence, widespread species are either regarded to represent truly widespread single species, or - more commonly - they are considered to represent complexes of multiple species, each with a more limited distribution (Camargo et al., 2006; Fouquet et al. 2007b). However, there is little evidence for both of these views so far and thus the status of populations remains largely unsolved. Of course, non-molecular reviews of old material - usually collected before the 20th century- revealed that at least specimens attributed to a single species collected from distinct biomes were in

fact distinct species (e.g. Barbour, 1909; Caramaschi and Pombal Jr, 2006; Narvaes and Rodrigues, 2009). But not all cases are that simple and some still are a taxonomic conundrum (e.g. Duellman, 1956, 1971).

Another example for such a putatively widespread amphibian species is *Dendropsophus minutus* (Hylidae), a small treefrog distributed east of the Andes from Colombia, Venezuela, Trinidad, the Guyana shield southward through Ecuador, Peru and Brazil to Bolivia, eastern Paraguay, Uruguay, and Argentina, up to 2000 m elevation (Frost, 2011). Although currently regarded to represent a single valid species, some studies noted differences and/or variation in colouration, osteology, advertisement calls and larval morphology (Cardoso and Haddad, 1984; Donnelly and Myers, 1991; Jansen et al., 2011; Kaplan, 1994; Murphy, 1997), and based on molecular analysis within a small part of the distribution some authors (e.g.Hawkins et al., 2007) suggest this nominal species to actually represent a species complex. However, due to rather limited datasets none of the studies so far was able to demonstrate the specific distinctness of certain populations. Taxonomy is further complicated by the fact that six additional species names are available, all currently regarded as junior synonyms of *D. minutus* (see Frost 2012). Additionally, apart from the nominal species and its synonyms, three other species possibly related to *D. minutus*, composing the *D. minutus* species group (see Faivovich et al., 2005), are difficult to differentiate from *D. minutus*.

In addition to the taxonomic importance and the related implications for conservation of a study on such a widespread and taxonomically complex taxon, the molecular analyses of the *D. minutus* complex and related species provide a singular opportunity for investigations on the biogeography of South America. As most Neotropical amphibian species are distributed within certain biomes, phylogeographical studies are often focused on specific regions whereas the global continental picture is scarcely studied (Crawford and Smith, 2005; Elmer et al., 2007; Noonan and Gaucher, 2005; Prado et al., 2012; Symula et al., 2003; Thomé et al., 2010). Because of the wide distribution of the nominal *D. minutus*, a phylogeographic study has the potential to integrate regional and continental scale analyses and to improve the understanding of South American biogeography. For instance, phylogeographical patterns from different regions of the continent can be analyzed simultaneously and further compared with previous studies, while the global continental pattern can be assessed.

As it is obvious that a clarification of the systematics and biogeography of such widespread nominal taxa requires a sampling including populations from all parts of its vast range, we made a collaborative effort bringing together as many *D. minutus* samples as possible, resulting in a coverage of most of the South American continent. Here, we present a first preliminary molecular approach at the continental scale with the goal to access the genetic differentiation and to obtain a first realistic

estimate of the number of divergent lineages hidden under the name *D. minutus* and related forms. Furthermore, we elaborate the distribution of these lineages throughout the continent. The goal of this first comprehensive study is not to fully resolve *D. minutus* species group systematics and phylogeography. We rather aim to highlight the genetic differentiation and biogeographic patterns, as well as its implications for future studies.

VI.II. Methods

VI.II.a. Data collection

We analyzed in this study 416 tissue samples of specimens identified in the field as *Dendropsophus minutus*, *D. aperomeus*, *D. delarivai*, *D. xapuriensis* or *D. stingi*. Genomic DNA extraction was carried out following standard salt extraction protocol (Bruford et al., 1992); using DNeasy Blood & Tissue Kit (Qiagen,Valencia, CA, USA); DNA extraction kit (Promega); or with standard phenol-choloform protocol implemented with an AutoGenprep 965 (AutoGen) automated DNA isolation robot. We used polymerase chain reaction (PCR) to amplify two fragments of mitochondrial DNA (mtDNA): one fragment of the 16S gene and the COI barcode fragment, using primers and annealing temperatures described in S table X. PCR products were cleaned using enzymatic purifications (Shrimp Alkaline Phosphatase and Exonuclease I). Sequencing of purified PCR products was performed with standard Sanger sequencing technique either by using ABI Big Dye V3.1 (ABI, Foster City, USA and Darmstadt, Germany) and resolved in on an automated sequencer at Genomic Engenharia corp. (São Paulo, Brazil) and Zoological institute of TU-Braunschweig (Braunschweig, Germany); or by sending to an external service platform (Macrogen, South Korea). Chromatograms were checked using the following softwares: Sequencer 4.5, MacClade 4.08, CodonCode Aligner v.3.x (CodonCode Corporation, Dedham, MA, USA).

VI.II.b. Gene tree inference and genetic distance calculation

Sequence alignment was performed using Muscle algorithm (Edgar, 2004) as implemented in the software MEGA5.0 (Tamura et al., 2011). Here and in subsequent analyses, substitution models were inferred using Akaike Information Criteria (Akaike, 1974) through the program jModel Test 0.1(Posada, 2008). To avoid overparametrized models we considered all models which had a value of delta AIC below five and chose the one with less parameters. Giving that resolving the systematic and phylogeny of *D. minutus* was not the aim of this study, and that COI and 16S fragments are both mitochondrial and can be considered linked, we favored numbers of samples over number of genes or sequence length and performed a gene trees inference using only the 16S fragment given that it

contained a more complete geographical coverage. The gene tree was estimated using Bayesian Inference with Markov Chain Monte Carlo (MCMC) algorithm in the software MrBayes 3.1.2.(Ronquist and Huelsenbeck, 2003). The 16S alignment was entirely used and gaps were treated as missing data. We ran two chains with different heating schemes, as set in the default of the program, for 100 x 10^6 iterations, sampling every 1000. We checked for parameter mixing, effective sample size (ESS) and convergence between runs using the program Tracer 1.5 (Rambaut and Drummond, 2009). The first 20% of the sampled trees were discarded as burn-in. Based on the generated topology we calculated uncorrected pairwise *p*-distances between major lineages and the mean *p*-distances within lineages with "complete deletion" in the software MEGA5.

VI.II.c. Phylogeographic Analysis

Recently, Lemey et al (2010) developed a phylogeographical method that incorporates geographical information on the tree inference and estimate dispersal and geographical distribution of internal nodes using continuous traits reconstruction through homogeneous Brownian diffusion (BD). The model is a Bayesian implementation of a model similar to the likelihood dispersal model implemented in the program Phylomapper (Lemmon and Lemmon, 2008). The employment of the model in a Bayesian framework is beneficial because topology uncertainty can be incorporated on the ancestral reconstruction and posterior density of node localities can be calculated (Lemey et al., 2010). Another improvement done by Lemey et al (2010) is the implementation of the Relaxed Random Walk model (RRW) which allows the dispersal rate to vary across branches. Using this approaches we performed a RRW phylogeographical analysis to estimate the geographical origin of the *D. minutus* group, possible dispersal routes, and approximately when it dispersed to different regions of the South American continent. We favored the RRW over the BD due to the wide geographical range of the samples analyzed. As some mtDNA lineages have wider ranges than others we assumed that a model that allow variation of dispersal rate would represent better our data. The method is implemented in the software Beast 1.7.x (Drummond et al., 2012) which estimates tree topology, node and root height and geographical locality of nodes simultaneously. In this and all analyses using Beast software we also used Beagle library v1.0 to improve computational efficiency. We used as locality traits of the terminals of the tree GPS coordinates collected in the field when available. When not available, we used as traits the coordinates of the municipality were the particular sample was collected, which was retrieved on online Gazetteers. For this analysis we used both mtDNA fragments concatenated excluding samples which one of the fragments was missing. We partitioned the sequences to allow different substitution rate and model for each gene while linking tree topology. As settings, a coalescent prior with constant size and an uncorrelated relaxed clock (Drummond et al., 2006), as well as HKY+G for both fragments and codon partition for the protein coding fragment linking the two first

codons was used. We ran three independent chains of 10^8 iterations with different random seeds sampling every 10000. In order to calibrate the tree we have estimated a substitution rate for the 16S fragment (see below). Mixing of parameter sampling, ESS and convergence were checked in Tracer 1.5. The resulted tree was summarized with Tree Annotator 1.7.x. The software SPREAD (Bielejec et al., 2011) was used to generate a *.kml* file which was plotted in a Google Earth map (http://earth.google.com).

VI.II.d. Substitution rate estimation

To estimate a substitution rate that could be used for the calibration of the phylogeographic analysis described above we constructed calibrated 16S gene tree with node constrains based on fossil and geographic evidence (Faivovich et al., 2005; Sanmartin and Ronquist, 2004; Wiens et al., 2006). We have downloaded 16S sequences of 216 Hylids, including all *Dendropsophus* species available on Genbank, and aligned with 16S sequences obtained in this study for *D. minutus*. Non overlapping regions were excluded to ensure that the fragment used in the substitution rate estimation and in the phylogeographic analysis were similar. Additionally we included one sequence representing each lineage of *D. minutus* group found here to simultaneously test the monophily of the group. We used a calibration scheme similar to what was used in previous studies of Hylids and other Amphibians (Lemmon et al., 2007; San Mauro et al., 2005; Smith et al., 2005; Wiens et al., 2006). We constrained three nodes with normal priors based on fossil evidence as follows: 1) most recent common ancestor (MRCA) of *Acris* and *Pseudacris* to 15Mya or older; 2) MRCA of *Hyla squirella* and *Hyla cinerea* to 15Mya or older; and 3) MRCA of all *Hyla* as 33Mya or older. The validity of the calibration number 3 can be contested (Faivovich et al., 2005). Therefore we performed separate analyses with and without calibration 3. The biogeographic evidence of the time of separation between South America and Australia was used to calibrate the divergence between Phyllomedusinae and Pelodryadinae. The complete separation of east Antarctica and Australia happen at about 35.5Mya, while the last landbridge between of South America and west Antarctica broke up at around 32-28Mya with the opening of the Drake passage and the formation of the South Circumpolar current (McLoughlin, 2001).We took into consideration two potential pitfalls of using this biogeographical evidence for calibration: 1) the divergence between the two groups could have predated the separation of the continents; 2) overseas dispersals may happen after land separation (Vences et al., 2003). Therefore we used a normal prior with 32Mya of mean and standard deviation of 6Mya to allow a higher probability for the MCMC to sample around that value (quantiles:5%=22.13; 95%=41.87) while avoiding hard boundaries. Before constraining the heights of the nodes we performed a preliminary run without constrain to verify if the resulting topology would recover the nodes correspondent to the calibration scheme. As this was confirmed we proceeded with the approach. The analysis was carried

out in the program Beast 1.7.x and it was repeated three times to check for convergence. We used a GTR+G+I mutation model inferred with jModelTest, a Yule Process speciation prior and an uncorrelated lognormal relaxed clock (Drummond et al., 2006). We ran a MCMC chain 10^8 iterations long, sampling every 1000. Effective sample size (ESS) mixing and convergence was checked in Tracer1.5. We used the posterior density of the parameter *ucld.mean* as an estimation of substitution rate for this 16S fragment of Hylids.

VI.II.e. Demographic analysis

To estimate the change in effective population size over time we performed a Bayesian Skyline Plot (BSLP) analysis using the program Beast 1.7.x. (Drummond et al., 2012). It is advisable to keep in mind that populations with strong genetic structure can experience different demographic processes. Therefore, as we found strong geographic pattern among the mitochondrial lineages, the analysis was performed for each lineage separately. Because multiples samples and reasonable geographic coverage are needed, we performed the analysis only for lineages represented by more than 18 sequences (Ho and Shapiro, 2011). As demonstrated by (Grant et al., 2012), the BSLP fails to recover the correct time of the demographic event mainly because when the substitution rates and node heights that are estimated using old fossil calibration are employed for the BSLP calibrations, they cause an underestimation of the spontaneous mutation rate at population level (Ho et al., 2011; Ho et al., 2005). Thus, they should not be used as calibration for population genetic inferences. Therefore we used the estimated mutation rate only to have a proxy for the relative comparison among BSLPs. To interpret the time of the demographic change we followed the arguments presented in Grant et al. (2012) and Ho et al. (2011). For this analysis we used both mitochondrial fragments, the complete 16S and the complete COI. As partition settings we unlinked substitution models and substitution rates for the two fragments and linked tree topology. The protein coding gene was partitioned to allow different rates in the third codon position. We fixed the *ucld.mean* of the 16S gene using the estimated mutation rate and let the program co-estimate a substitution rate for COI. Based on preliminary analysis that showed no strong variation of substitution rate across branched (*stdv.ucld* posterior density included zero) we assumed a strict molecular clock. Each analysis was ran long enough to ensure effective sample sizes (ESS) higher than 200,000 for all parameters, which was accessed using Tracer 1.5 (Rambaut and Drummond, 2009).

VI.III. Results

The resulting alignment of the 16S fragment contained 416 samples and it was 489bp long including gaps, while the COI fragment contained 340 samples with 591bp long. All Bayesian analyses performed in this study yielded converging results among independent runs with overlapping posterior densities of estimated parameters and high values of effective sample sizes (ESS). The 16S gene tree shows several mitochondrial lineages tentatively placed in 19 groups named from A to S (**Figure 8, Figure 9**). Lineages A-J present in general a more widespread distribution and a relatively lower genetic divergence while lineages K-S show relatively more restricted distribution and deeper divergence (**Table 3**). The *Dendropsophus* group was recovered as monophyletic. The clade containing lineges A-J show high posterior probability (**Figure 8, Figure 10**) and is defined here as the *D. minutus* complex, as J contains samples from the type locality of *D.minutus*. The other clades represent close related species part of the *D.minutus* group which was also recovered as monophyletic with high node support (**Figure 10**).

The distribution of mitochondrial lineages is largely allopatric, evidencing the genetic structure and the strong geographical pattern. Nevertheless some cases of simpatry are present (**Figure 9**). The estimated substitution rate for the 16S fragment was 7.35×10^{-3}/site/Ma (median of *ucld.mean* parameter [95%HPD = $6.1 – 8.7 \times 10^{-3}$]). The exclusion of calibration number 3 (see methods) did not change substantially the estimates of substitution rate.

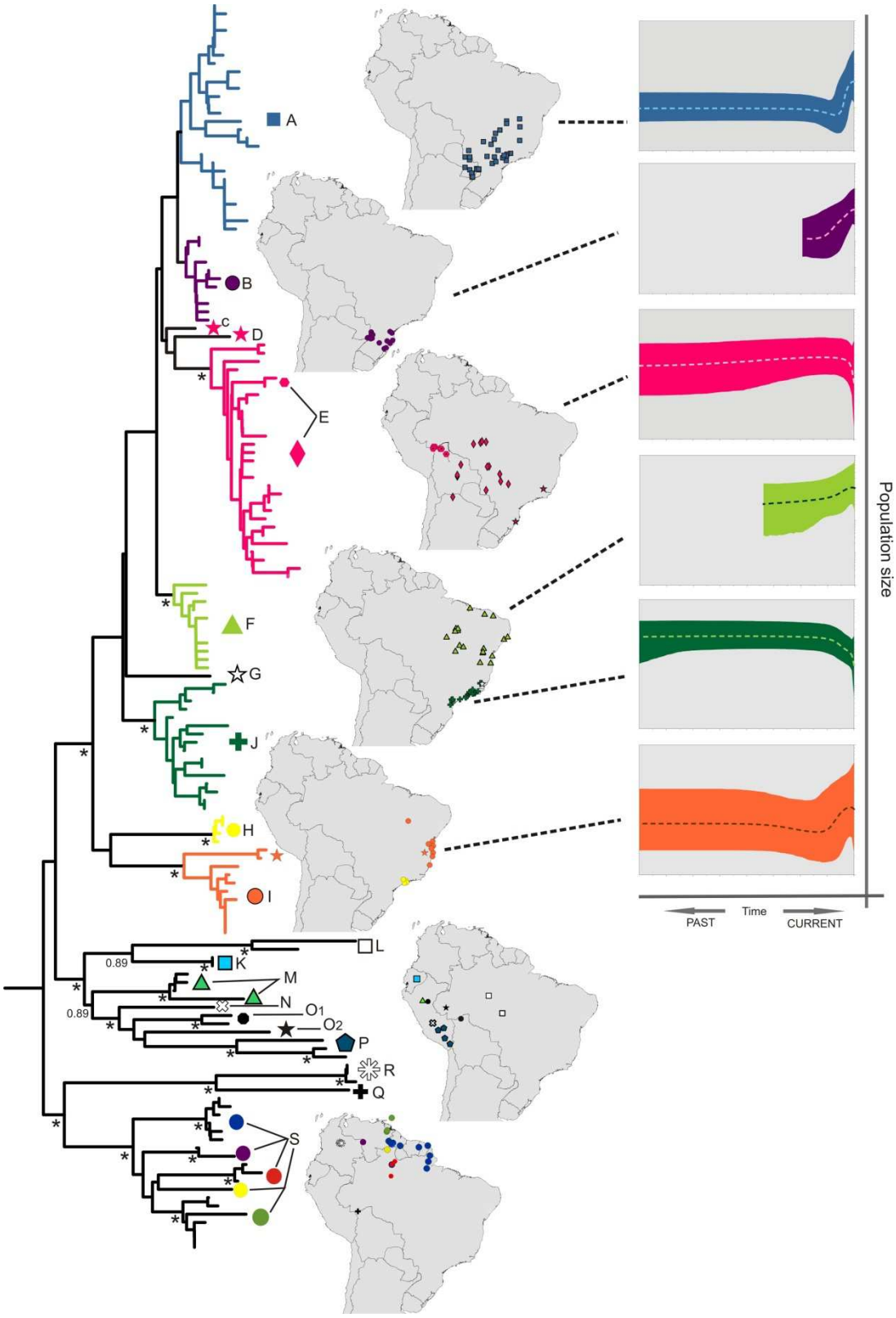

Figure 8: 16S Minimum Evolution tree calculated in MEGA 5 with collapsed haplotypes for easy visualization; distribution maps of mtDNA lineages and Bayesian Skyline Plots (BSLP). Node supports are derived from the Bayesian phylogenetic inference performed in software mrBayes. Asterisks represent nodes with probability equals to 1. Probabilities lower than 0.8 are not shown.

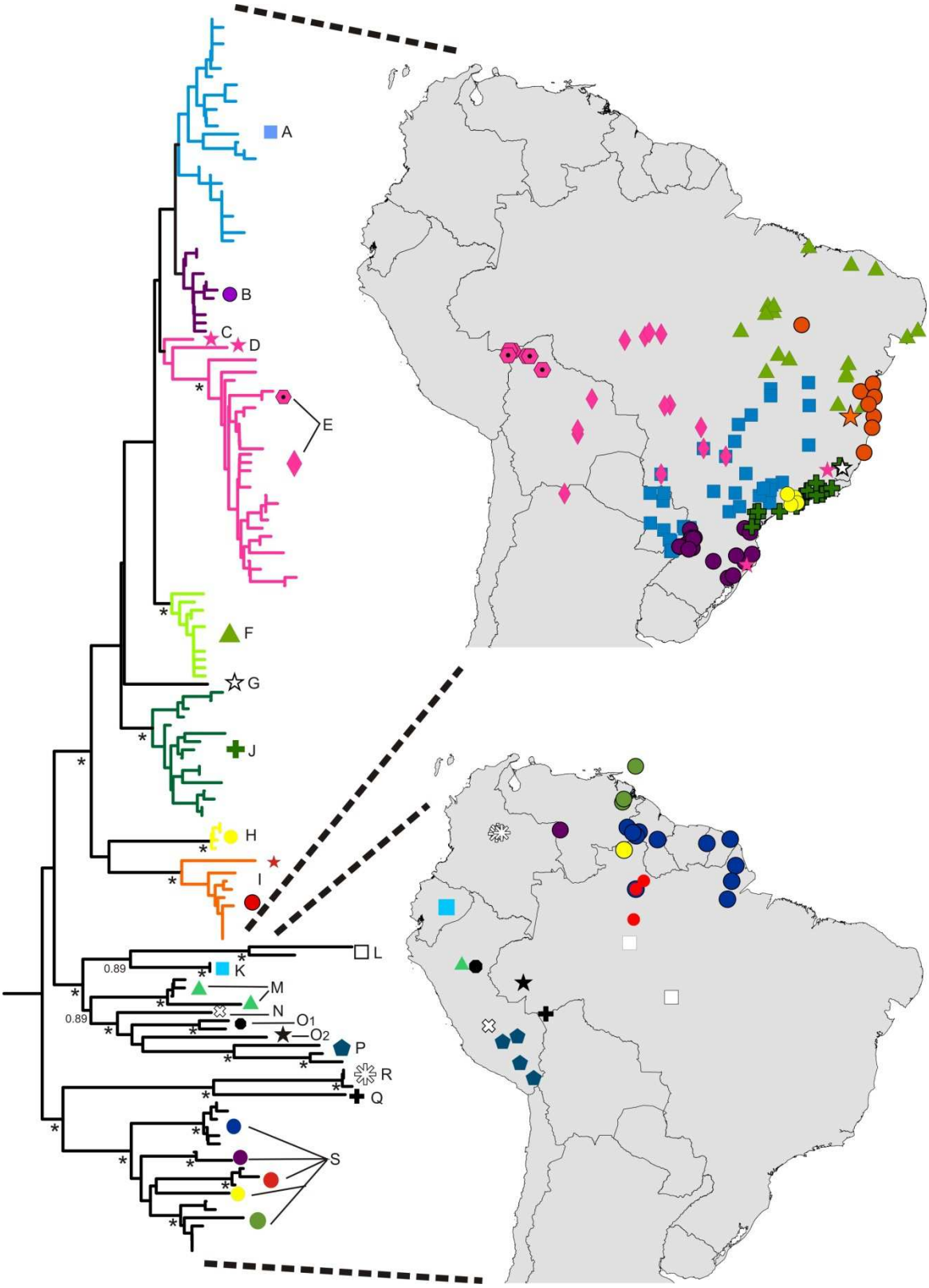

Figure 9: 16S Minimum Evolution tree constructed in MEGA 5 with collapsed haplotypes for easy visualization; distribution maps of mtDNA lineages showing localities where lineages occur in simpatry. Node supports are derived from the Bayesian phylogenetic inference performed in software mrBayes. Asterisks represent nodes with probability equals to 1. Probabilities lower than 0.8 are not shown.

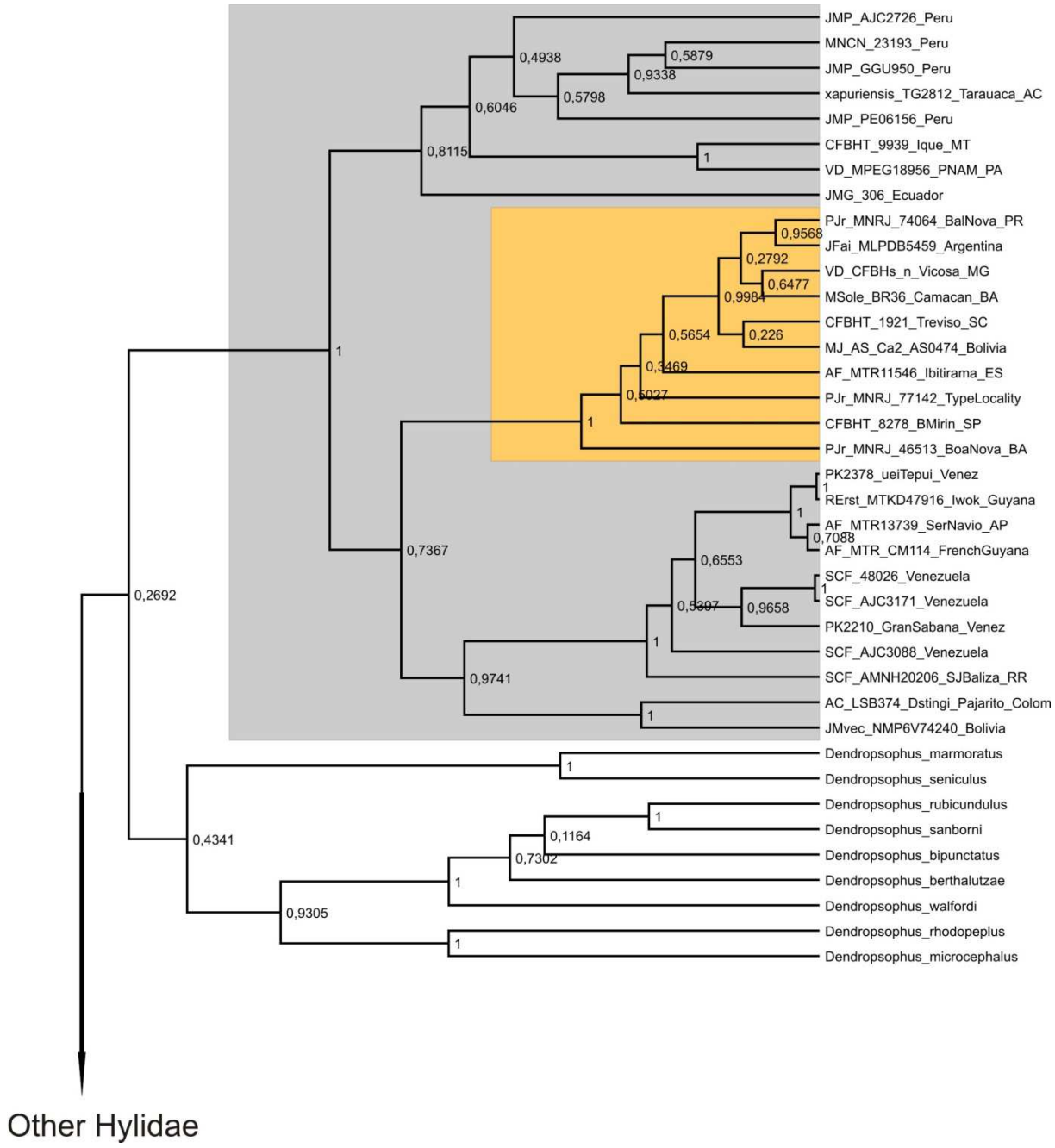

Figure 10 50%-majority rule consensus tree derived from Bayesian inference analysis of 216 16S sequences of Hylidae plus *D. minutus* group, that was performed for the substitution rate estimations using the program Beast1.7.x. The *Dendropsophus minutus* group is highlighted in grey and the *Dendropsophus minutus* complex in orange. Node numbers indicate posterior probabilities.

Table 3: Pairwise uncorrected mean *p*-distances between lineages and mean *p*-distances within lineages. (n/c) Lineages that are represented by one sequence.

	Pairwise uncorrected mean *p*-distance																			Within group mean *p*-distance	
	A	B	E	C	D	F	G	J	H	I	L	K	M	N	O1	O2	P	R	Q		
A																				0.0132	A
B	0.017																			0.0034	B
E	0.041	0.033																		0.0130	E
C	0.024	0.016	0.029																	n/c	C
D	0.033	0.024	0.034	0.023																n/c	D
F	0.026	0.024	0.033	0.023	0.030															0.0044	F
G	0.043	0.038	0.048	0.037	0.045	0.041														n/c	G
J	0.045	0.041	0.053	0.044	0.050	0.041	0.044													0.0139	J
H	0.065	0.060	0.070	0.059	0.062	0.058	0.061	0.056												0.0015	H
I	0.065	0.053	0.071	0.058	0.067	0.062	0.053	0.055	0.057											0.0080	I
L	0.106	0.103	0.113	0.104	0.104	0.101	0.103	0.109	0.110	0.102										0.0264	L
K	0.079	0.074	0.083	0.076	0.076	0.080	0.071	0.075	0.098	0.077	0.073									0.0000	K
M	0.090	0.083	0.082	0.081	0.084	0.087	0.087	0.081	0.089	0.084	0.084	0.078								0.0145	M
N	0.081	0.072	0.075	0.068	0.076	0.076	0.073	0.079	0.076	0.082	0.089	0.082	0.063							0.0000	N
O1	0.082	0.078	0.076	0.075	0.083	0.078	0.085	0.085	0.096	0.091	0.095	0.068	0.079	0.055						0.0141	O1
O2	0.092	0.084	0.086	0.079	0.088	0.088	0.088	0.095	0.099	0.089	0.091	0.073	0.075	0.048	0.052					n/c	O2
P	0.110	0.104	0.109	0.098	0.108	0.109	0.098	0.107	0.115	0.101	0.107	0.069	0.091	0.078	0.072	0.072				0.0183	P
R	0.115	0.114	0.115	0.117	0.108	0.107	0.117	0.120	0.123	0.118	0.118	0.108	0.114	0.108	0.117	0.111	0.127			0.0015	R
Q	0.114	0.113	0.117	0.116	0.107	0.107	0.116	0.114	0.127	0.116	0.121	0.113	0.118	0.124	0.124	0.127	0.138	0.072		n/c	Q
S	0.082	0.079	0.091	0.081	0.072	0.079	0.085	0.080	0.076	0.076	0.099	0.075	0.085	0.084	0.085	0.102	0.100	0.101	0.105	0.0301	S

The phylogeographical analysis (RRW) suggest a central Amazonian origin for the *D.minutus* group (**Figure 11A**), with further dispersion to the Brazilian Atlantic Forest (AF), Guyana shield and the Andean region (**Figure 11 B and C**). Furthermore the results suggest a recent southern dispersal route between AF and Amazonia and a recent colonization of southern AF and eastern Paraguay as well as northeastern and central Brazil and Guiana shield (**Figure 11 C and D**). The Bayesian skyline plots show a strong recent expansion of lineage A, while weak expansion sign for lineages B, F and I. Lineages E and J show some sign of population shrinking (**Figure 8, Figure 12 A**). However, considering the confidence interval, constant population size can only be rejected for lineage A. The coalescence time of each of the lineages analyzed with the Bayesian skyline model corroborate the phylogeographical analyses (RRW), where lineages B and F, distributed in the south and northeast of Brazil respectively, show a more recent coalescence when compared to lineages A, E, I, J. Lineages E and I show the deepest coalescence while A and J show intermediate coalescent times (**Figure 12**).

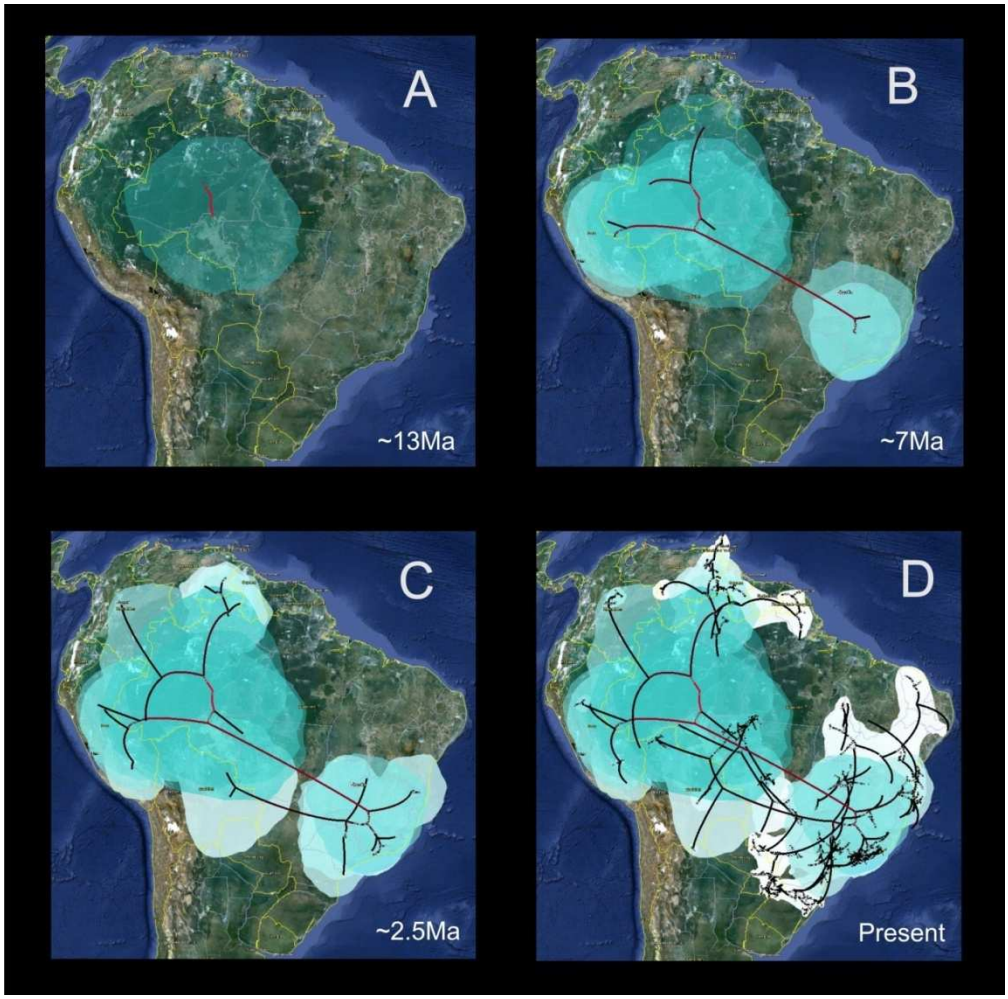

Figure 11: Phylogeographical reconstruction using the Relaxed Random Walk model (RRW). A) center of origin of the *D. minutus* group. B) Dispersal to west Amazonia, Guiana shield, Andean region of Peru and eastern Brazil. C) Dispersal from east Brazil to lowland f Bolivia. Further dispersal to Guiana shield, Peruvian and Colombian areas. D) Recent dispersals to northeast and south Brazil, east Paraguay and Guiana shield. Green polygons and red branches indicate old events while pale polygons and black branches indicate recent events.

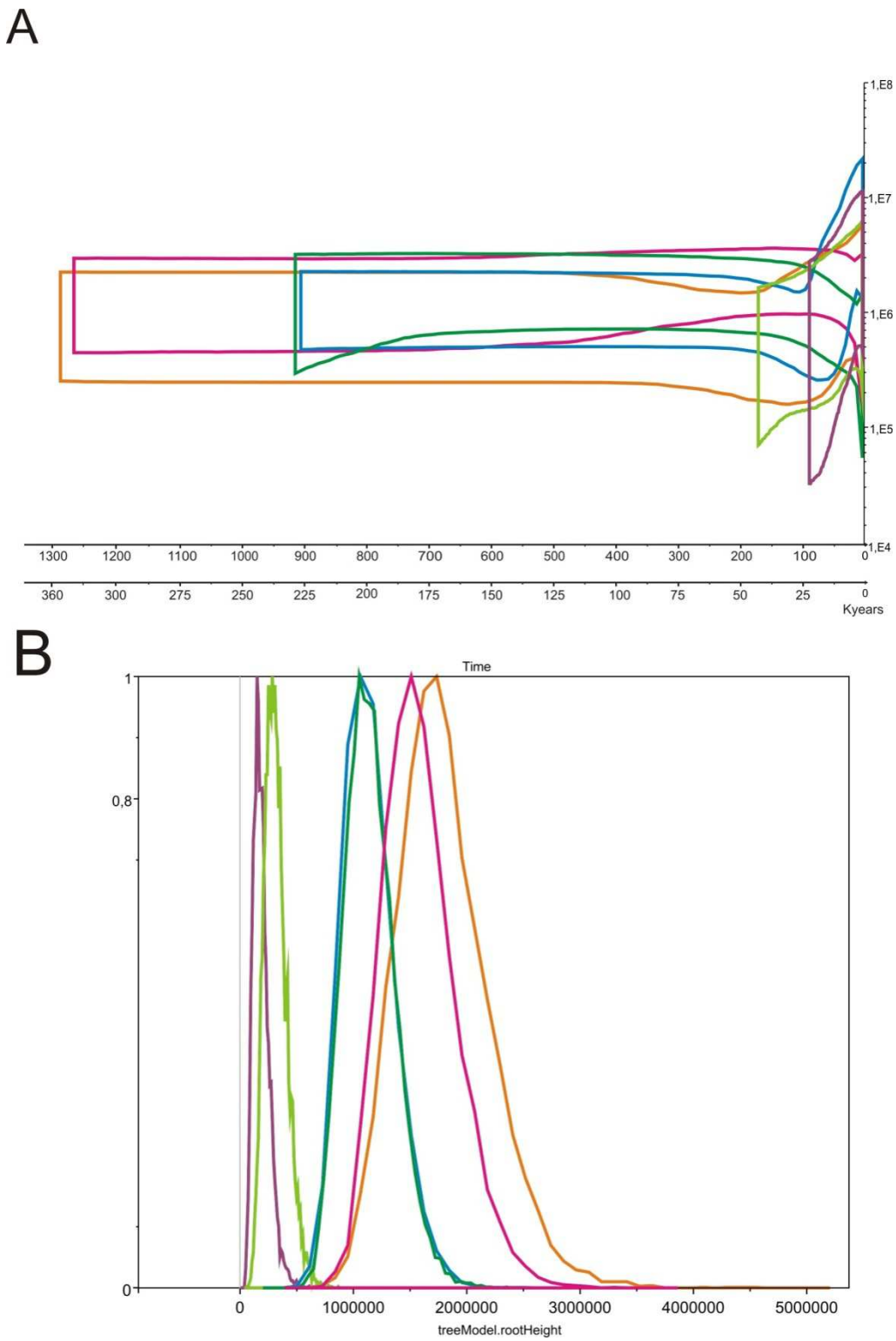

Figure 12 : A) Combined confidence intervals of the Bayesian Skyline Plot Results calibrated with the estimated 16S mutation rate (upper time axis) and alternative arbitrary mutation rate (lower time axis – see discution for additional information). The left ends of the BSLPs are represented by the lower limit of the 95%HPD of the root.height (most recent probable coalescent time). B) Relative marginal posterior densities of the root.Height for each lineage analyzed under a Bayesian Skyline plot model, showing the relative differences in coalescence times for each mtDNA lineage. The colors represent each lineage analyzed. Blue: lineage A; purple: lineage B; pink: lineage E; light green: lineage F; dark green: lineage J; orange: lineage I.

VI.IV. Discussion

VI.IV.a. Taxonomic implications

Given the numerous divergent lineages and the degree of differentiation revealed by our analyses, it is not presumptuous to state that very likely several additional species are hidden under the name *Dendropsophus minutus*, and that additional species are yet to be described within the *D. minutus* species group. At the current stage we refrain from any formal taxonomic decision, as the clarification of the taxonomic status of the populations involved requires a thorough approach integrating more datasets on nuclear DNA, morphology and behavior (calls), as well as the examination of crucial type specimens. Nevertheless, some interesting indications can be derived from our results that are worth to be discussed.

Regarding the *D. minutus* complex (*i.e. D. minutus* sensu lato), our analyses revealed ten divergent clades (lineages A–J; **Figure 8**). Although the specific distinctness of these lineages cannot be addressed here, each of these clades could represent a divergent evolutionary lineage, given their degree of differentiation and their generally allopatric occurrence. Even within the mentioned clades, further differentiation at the species level seems to be possible. For example, populations forming the northern Bolivian subclade (pink hexagons **Figure 8**, **Figure 9**) within lineage E apparently differ constantly in their advertisement call characteristics and larval morphology from other populations of the lineage distributed in the eastern lowlands of Bolivia and the Cerrado region of Brazil (Jansen et al. 2011; unpubl. data). Such differentiation within lineage may also be expected for other major clades of the *D. minutus* complex, if more data on morphology and calls accumulate. Given this complex situation, it seems possible that some of the available names currently regarded as synonyms of *D. minutus* from which topotypic material is included in our analyses have to be revalidaded in future taxonomic revisions. This scenario is likely for the names *Hyla bivittata* and *H. emrichi* (clade B), and *H. velata* (clade E). On the other hand, topotypic samples from close to the type localities of *H. pallens* and *H. suturata* cluster with samples of topotypic *D. minutus*, thus seemingly confirming their status as junior synonyms.

Concerning other species related to *D. minutus* sensu lato (mostly also treated as populations of *D. minutus* in the literature), our analyses revealed an even more complex pattern of differentiation (clades K–S; **Figure 8**, **Figure 9**). Considering these lineages to represent members of the *D. minutus* species group, we may state that the following nominal taxa are likely part of it: *D. aperomeus*, *D.* cf. *delarivai*, *D. stingi* and *D. xapuriensis*. For these species, relationships were assumed by morphology only and in two cases (*D. aperomeus*, *D. stingi*) the species were not allocated to the *D. minutus* species group before (Duellman, 1982; Faivovich et al., 2005; Kaplan, 1994; Köhler and Lötters, 2001; Martins and Cardoso, 1987). Among clades K–S, differentiation is well-pronounced and it is

obvious that this group contains several formerly unrecognized divergent evolutionary lineages. Being conservative, we might suspect that clades K–S contain nine species, namely four already known ones and five undescribed taxa. Similarly to the *D. minutus* complex, it seems likely that one available name, *Hyla goughi* (Trinidad and Tobago), has to be removed from the synonymy of *D. minutus*. Given the considerable differentiation present within some Andean clades, or among samples originating from the Guyana shield, this number might rather be an outraged underestimation.

In summary, we conclude that indeed several species are hidden under the name *Dendropsophus minutus*. When referring to the *D. minutus* complex alone, about ten to fifteen species might actually be involved. When referring to the whole *D. minutus* species group, our results indicate that about twenty to thirty-three species might be considered if in the future we conclude that each mitochondrial lineage truly represent a different species. Although our analyses were not adequate to clarify these taxonomic issues, it became quite clear that the current taxonomy, considering four species within the *D. minutus* species group only (Faivovich et al. 2005), does not properly reflect the actual complexity of the evolutionary history of this group of frogs.

VI.IV.b. Biogeography

The region of possible origin of the *D.minutus* group is represented by the polygon and branching (**Figure 11 A**). The centroid corresponds to the central Amazonian basin, but the polygon also overlaps part of western Amazonia and part of Brazilian shield. The estimated time frame of coalescence of all mtDNA lineages [15.0 Ma, (95%HPD, 11.0 – 18.0Ma)] fell into middle to late Miocene, a period of high rates of Andean mountain building (~12Ma), when the Western Amazonia was characterized by a large wetland formation of shallow lakes and swamps, the Lake Pebas System (Hoorn et al., 2010). This time coincides with a peak of diversity of Amazonian plants (~13Ma) according to the pollen record, as well as with early diversification of some Amazonian amphibians and mammals, pre-montane birds and plants (Hoorn et al., 2010). Interestingly, it coincides with the diversification of the *Allobates trilineatus* complex (14.0 – 15.0 Ma), which started within the Amazon basin (Santos et al., 2009); and with *Rinella marina* complex (10.7 – 17.2Ma) which likely has an Amazonian origin (Vallinoto et al., 2010). The initial diversification of Amazonian gecko of the genus *Leposoma* (around 13.9Ma) also falls into the same period (Pellegrino et al., 2011). The RRW model suggest dispersal to the Andean area and Guiana shields after the initial diversification, between 8.0 – 10.0Ma (**Figure 11 A and B**), with additional recent dispersals (**Figure 11 C and D**). This is also in agreement with Santos et al (2009) who suggested a wave of dispersals of ancient Dendrobatidae lineages from the Amazon basin to the same regions, approximately in the same period (8.8 – 10.8Ma), followed by another more recent wave (0.7 – 6.0Ma).

Another interesting pattern recovered by our analysis is the dispersal from Amazonia to east Brazil and vice-versa (**Figure 11 B, C and D**). An old relationship between Amazonian and Atlantic Forest amphibians has been recently reported, and an Amazonian origin has been suggested (Canedo and Haddad, 2012; Fouquet et al., 2012a; Fouquet et al., 2012b; Heinicke et al., 2007). Some authors propose the formation of a diagonal band of open and dry biomes in the Eocene (currently Caatinga, Cerrado and Chaco biomes) separating the Atlantic Forest from Amazonia as the main paleogeological event that likely caused ancient vicariance of some amphibian lineages. Given this evidence and considering the amphibian dependence of water and moisture (Vences and Köhler, 2008), it is reasonable to assume that the 'dry diagonal' acts as a dispersal barrier for most amphibians, particularly for forest dependent taxa, and that species distributed in both Atlantic Forest and Amazonia represent complexes of generalist species. Despite the evidence of an old relationship between Amazonia and Atlantic Forest, biogeographical analysis of different vertebrate groups as well as climatic and floristic evidence suggest relatively recent dispersal corridors between these isolated forested areas (Cabanne et al., 2008; Costa, 2003; Melo Santos et al., 2007; Wang et al., 2004). On the other hand the phylogeography of species adapted to dry biomes suggests past connectivity between open area formations currently isolated (Adrian Quijada-Mascareñas et al., 2007; Wuster et al., 2005). The idea of dispersal corridors and temporary barriers gain support when paleoclimatic models of Atlantic Forest and Cerrado (a 'dry diagonal' biome) are considered (Carnaval and Moritz, 2008; Werneck et al., 2012b). The models predict large areas of unstable climate suitability for both biomes when projections to different past climate conditions are overlapped. If correct, they suggest the potential of a dynamic process of landscape change through time. The time we found for the first dispersion of ancient *D. minutus* from Amazonia to eastern Brazil (7 – 10 Ma) is much more recent than the reported period for other Amphibian groups (Fouquet et al., 2012b; Heinicke et al., 2007). Possibly the dispersal is even more recent than what was found because the age of the nodes close to the tip of the tree are probably overestimated (see Ho et al. 2011). Nevertheless, we feel discouraged to assume that a corridor of forest was necessary for the dispersion of *D.minutus* group to the east. Frogs from the *Dendropsophus minutus* complex and related species are pond breeders that can be found in a variety of habitats (Frost 2012). They are currently found in savanna formations and deciduous forests, which indicate that at least some populations have a high tolerance to seasonality. Therefore, dispersal seems possible without forest continuity. However, with the dataset and results presented here it is difficult to speculate a possible route for the first dispersion to the east. Only a long branch connects the polygons of the center of origin and the eastern arriving area, demonstrating that the history of the initial dispersal route cannot be recovered with this dataset.

The polygon in the east (**Figure 11 B**) correspond to the *D. minutus* complex node, as defined here (lineages A – J), suggesting an eastern origin for these lineages. Possibly, after dispersion to the

east there was a vicariant event that isolated the ancestor of *D. minutus* complex from Amazonian lineages. Interestingly, the polygon that suggests the probable area of origin of *D. minutus* complex encompasses potentially climatic stable areas within the Atlantic Forest (Carnaval and Moritz, 2008). Thus, it is possible that after east dispersion there was one or more periods when the distribution of the ancient *D. minutus* was close to areas of climatic stability while populations of other regions were extinct and the mitochondrial history along the route was lost. From the area of origin the mtDNA lineages of *D. minutus* diversify and the complex disperse to other areas of east South America, central Brazil and Amazonia, resulting in a pattern that evidences a southern dispersal route between Amazonia and Atlantic Forest (**Figure 11 C and D**). This southern route was proposed as one of the floristic connection pathway between Atlantic Forest and Amazonia (Por, 1992) and the analysis of some species of small mammals corroborate this hypothesis (Costa, 2003). Moreover, the palinological record from different continents of the southern hemisphere suggest the existence of a band of moisture at this latitude (~23°S) during the Last Glacial Maxima (LGM) (Ledru et al., 2005), which raises the possibility that the same phenomena occurred in older periods if similar paleo-climate conditions were present. Currently, the area along the pathway has an interesting arrangement of biomes. Before human deforestation, the AF domain was formerly distributed towards the west in its southern portion. From this western part of AF towards northwest a narrow stretch of savanna (~250 km in a straight line) separates AF from the wetlands (Pantanal), which are in contact with the Amazonian border. Hence, a mosaic of forest fragments and open area in combination with the existence of a wetland formation might facilitate dispersion of flexible species, promoting faunal exchange between separated forested biomes.

VI.IV.c. Phylogeography of Dendropsophus minutus complex

Within the Atlantic Forest (AF) domain the distribution pattern of the lineages recovered two discontinuities/contact zones also found for other AF vertebrates. One at the south of Sao Paulo state, represented by the southern limit of the distribution of lineage J, northern limit of lineage B; and another at Espirito Santo state, northern limit of lineage J, southern limit of lineage I (**Figure 8, Figure 9**). A similar southern genetic break was found, for instance, for pit-vipers from the *Bothrops jararaca* complex (Grazziotin et al., 2006) and toads from the *Rhinella crucifer* complex (Thomé et al., 2010). The northern break was also found for *Rhinella crucifer* complex (Thomé et al., 2010), geckos from the *Gymnodactylus darwinii* complex (Pellegrino et al., 2005), the crab eating fox, *Cerdocyum thous* (Tchaicka et al., 2007) and the lesser woodcreeper, *Xiphrorhincus fuscus* (Cabanne et al., 2008). The causes of these genetic breaks are largely debated, authors propose different none mutually exclusive hypothesis to explain these patterns, but the topic is still open to speculations (De Mello Martins, 2011). Interestingly we found deep coalescence and/or sympatry of mtDNA lineages

within or close to proposed AF pleistocenic refugia (Lineages H and J; lineage I, **Figure 8**, **Figure 12 B**) (see (Carnaval et al., 2009; Carnaval and Moritz, 2008), which can be seen as an indication that these genetic breaks are an effect of climatic oscillations. Although some studies suggest that the Pleistocene refugia model (Haffer, 1969) alone cannot explain the genetic pattern of some vertebrate species (Costa, 2003; Grazziotin et al., 2006; Thomé et al., 2010), Carnaval et al. (2009) demonstrated that climatic oscillations indeed shaped the current genetic pattern of Atlantic Forest amphibian species. Moreover, even considering older geological events as having explanatory potential for current phylogeographical patterns, no study so far fully rejects the Pleistocene influence on within species processes and population differentiations (Cabanne et al., 2008; Grazziotin et al., 2006; Prado et al., 2012; Thomé et al., 2010; Werneck et al., 2012a).

We found demographic expansion for lineage A starting at about 100 Ka (**Figure 12 A**). This lineage is co-distributed with another Hylid, *Hypsiboas albopuctatus,* and Prado et al (2012) also found recent population expansion for this species. The palinological evidence from the area where these two species occur, which is currently dominated by a mosaic of semi-deciduous forest and cerrado, show that during the last glaciation (50-18Ka aprox.) the area was covered predominantly by grasslands (Behling, 1998; Ledru et al., 1996). The plant composition was similar to what is currently found in Patagonian savannas (Ledru et al., 1996), indicating a very cold and dry climate. In one of the localities Behling (1998) suggest winter temperatures of -5°C at 755m above sea level. Moreover, the absence of *Araucaria* trees indicates a very dry climate. In another area Ledru et al. (1996) found no evidence of aquatic vegetation in the pollen record between 50 – 40Ka (Ledru et al., 1996). Furthermore, paleomodels show contraction of both Cerrado and AF biomes for the LGM (Carnaval and Moritz, 2008; Werneck et al., 2012b). This does not rule out the possibility that favorable conditions for *D. minutus* were present, particularly at lower elevations, but makes rather unlikely that an increase in population size occurred from LIG to the present. Conversely, such conditions would agree with a decline in population size. This led us to speculate possible reasons for our finding of population expansion for that period.

The dating of demographic changes is extremely prone to error and can result in misinterpretation of the data (Grant et al., 2012). A time-dependence of molecular rates has been reported given the evidence that mutation rates calculated in pedigree studies largely exceeds those estimated using phylogenetic inference (Ho et al., 2011). In some cases the mutation rate at population level can be more than an order of magnitude higher than the phylogenetic substitution rate (Ho et al., 2011). Taking that into account we propose an alternative interpretation for the time of the expansion found in this study. Using an arbitrary mutation rate (~3.0×10^{-2}/site/Ma) 4 times faster than the one estimated here for the 16S fragment, the date of the demographic expansion would have happened after the Last Glacial Maxima (LGM) (**Figure 12 A**). This means that recent climatic change can be

used as explanation for the population expansion, which is in this case more reasonable, particularly if we assume a decline in Ne after the Last Inter-Glacial (LIG). It is important to consider that the information about population history imprinted in the mitochondrial genealogy is more prone to be erased when there is decline in population size (Ne) because the Ne of the mtDNA is four times smaller. In addition, one-locus demographic analysis cannot reconstruct events prior to the last bottleneck for this particular locus (Grant et al., 2012). It is then more plausible to assume a Ne increase after LGM when temperature and humidity increased and the semi-deciduous forest and Cerrado expanded (Behling, 1998; Carnaval and Moritz, 2008; Ledru et al., 1996; Werneck et al., 2012b).

The signs of population expansion for lineages B and F agree with the idea of recent colonization of those areas, which is corroborated by the relatively recent coalescence of these lineages (**Figure 12**). BSLPs of lineages J and E showed signs of population contraction. This can be tentatively explained by the recent expansion of the ombrophilous forest which is probably not as suitable a habitat for *D. minutus* as a mosaic of forest and open area. However considering the confidence interval of the BSLPs we cannot reject constant population size for all these lineages. Large confidence intervals can be reduced with the increase of loci in the analysis (Heled and Drummond, 2008). Also, there is the possibility that some populations are more adapted to forest while others are to open environments. Therefore, more data is needed before we take any other conclusions about the historical demography of *D. minutus* complex.

VI.V. Conclusions

This study present interesting results that are important for the understanding of South American biogeography. Our study confirms the idea that biological diversity is largely underestimated in the tropics. Nevertheless, the results should be taken cautiously, given that our analysis is based exclusively on mtDNA and the data represent a partial view of the entire picture, which can change if other loci are analyzed. In addition, there has been an increase of reports of incongruence between biogeographical pattern of mtDNA and Nuclear DNA in animals (Toews and Brelsford, 2012). At this point we are unable to assign any of these mitochondrial lineages to valid species. Further studies are necessary to confirm and refine the biogeographic conclusions as well as to resolve the taxonomy of the group. Our work sheds the essential first light that is a starting point for deeper investigations of the taxonomy of the *Dendropsophus minutus* complex and related evolutionary processes.

VII. Finding stable areas with genes: comparative phylogeography of Brazilian Atlantic Forest species.

Abstract

The Brazil's Atlantic Forest (AF) is a species-rich tropical region with high levels of endemism. In the recent years attention has been given to this tropical region, and researchers have been proposing different diversification mechanism to explain its diversity pattern. One of the most debated hypothesis is the Refugia Model (RM). In the recent years studies have been showing evidence to reject and support the hypothesis. Given that, there is no consensus in the literature about the processes through which the diversity and phylogeographical patterns emerged. One aspect that weakens the RM is the existence of high genetic diversity for some population of species distributed in areas that should present low genetic diversity according to the model. Like the southeast AF, a region located south of the predicted stable area. Distribution data alone, as well as molecular analysis of some AF species support an endemism area for this region suggesting that it has been stable during climatic oscillations. Comparative phylogeography has the potential to uncover the existence of a common biogeographic history among co-distributed taxa. The continuous phylogeographical analysis recently implemented in the software Beast 1.7 can perform a phylogeographical reconstruction of a particular genealogy where geographical origin of nodes can be estimated. In order to perform a comparative analysis of AF species we downloaded from the GenBank mitochondrial sequences of six well sampled AF species, five amphibians and one snake. Additionally we have generated data for three other co-distributed frog species to be included in the analysis. According to the phylogeographic analysis deep mitochondrial sister lineages have been originated apart from each other and away from contact zones. Furthermore we found high overlapping frequency of center of origins of co-distributed lineages at two regions of the studied area: one region at the Center-North AF, which agrees with the recently proposed RM; and another at the Southeast AF, which agrees with floristic studies that propose a stable area within that region. Our results support climatic oscillations and related refuges as likely explanation for the currrent philogeographical pattern of Atlantic forest species.

VII.I. Introduction

The origin of the tropical diversity has puzzled the minds of biologists since the 19th century (Wallace, 1852). Since then, different diversification hypotheses were postulated. These hypotheses can be divided roughly into two main categories: i) allopatric, proposing isolation caused by rivers or Pleistocene refugia and consequently diversification; ii) non allopatric, which suggest that differentiation occurred by diversifying selection across environmental gradients (Moritz et al., 2000). Formerly, those biogeographical hypotheses were difficult to test because distribution data alone accommodates multiples interpretations (Endler, 1982a, b; Moritz et al., 2000). With the birth of phylogeography and further increase in computing power and development of analytical tools, testing of evolutionary hypotheses has become feasible (Avise et al., 1987; Fagundes et al., 2007; Schneider et al., 1999). Currently, evolutionary biologists rely on DNA analysis, distribution patterns, peleomodeling, computer simulations and other tools to investigate diversification processes. Molecular data of different organisms across different regions has accumulated over the years, however the southern hemisphere tropical biota still poorly studied when compared with northern hemisphere temperate regions (Beheregaray, 2008; Köhler et al., 2005). One of these poorly studied tropical regions that recently have received attention from the scientific community is the eastern Brazilian region and its costal rain forest.

The Brazil's Atlantic Forest (AF) holds more than 20,000 plant species and more than 2,000 vertebrate species, of which around 40% are endemic (Haddad et al., 2008). It is one of the most threatened ecosystems of the planet, being identified as one of the five major biodiversity hotspots for conservation (Myers et al., 2000). Although its high biodiversity has been recognized, studies to understand diversification processes in the AF are still in its infancy. Currently there is no consensus in the literature about the processes through which the diversity and phylogeographic patterns emerged (De Mello Martins, 2011). One of the most debated diversification hypotheses is the Refugia Model (RM). The refugia hypothesis is not new (Haffer, 1969), but only recently niche and climatic modeling has allowed explicit testing for congruence between molecular data and putative modeled refugia (Carnaval et al., 2009; Graham et al., 2006; Graham et al., 2004). While some authors observe congruence between phylogeographic patterns of AF fauna and the RM (d'Horta et al., 2011; Fitzpatrick et al., 2009), others find poor accordance and therefore invoke other hypotheses to explain genetic breakes and divergence among lineages (Thomé et al., 2010). For instance, the riverine hypothesis has been used to explain a south-north discontinuity found in AF species that agrees with the current position of the Doce River (**Figure 13**) (Brunes et al., 2010; Pellegrino et al., 2005; Tchaicka et al., 2007; Thomé et al., 2010). Another common break at south of Sao Paulo State is close or coincide with the Guapiara Lineament and Paranapanema River (Amaro et al., 2012; Brunes et al., 2010; Cabanne et al., 2007; d'Horta et al., 2011; Grazziotin et al., 2006; Thomé et al., 2010).

Quaternary tectonics, Pliocene tectonics and marine introgressions have also been suggested as possibly related to genetic breaks (De Mello Martins, 2011; Grazziotin et al., 2006; Thomé et al., 2010). In addition, some studies show evidence of faunal exchange between AF and Amazonia, suggesting that the AF cannot be considered as a natural biogeographic unity but rather as a composite of species with different biogeographic histories (Costa, 2003; Fouquet et al., 2012b). Because these hypotheses are non-mutually exclusive, a combination of all these factors has been proposed (Costa, 2003; De Mello Martins, 2011; Fouquet et al., 2012b; Grazziotin et al., 2006; Thomé et al., 2010). Indeed, a unique diversification model seems too simplistic to explain the complex patters found in AF and other tropical regions in general. Even though, researchers still try to find if there was a major diversification force that generated the current diversity pattern (Carnaval et al., 2009).

Comparative phylogeography has the potential to uncover the existence of a common biogeographic history among co-distributed taxa (Arbogast and Kenagy, 2001; Bermingham and Moritz, 1998). The comparative analysis of three amphibian species, carried out by Carnaval et al (2009), coupled with the paleomodeling performed by Carnaval and Moritz (2008), demonstrated that stable areas maintained high genetic diversity while unstable areas were recently colonized. The authors identified a large potentially stable area north of the Doce River (**Figure 13**). However, as pointed out by Carnaval and Moritz (2008) themselves, their results contrast with the phylogeographical patterns of some species that show high genetic diversity south of Doce River. As an explanation for the incongruence they suggested "*suboptimal model performance in areas of steep altitudinal gradients"*. In fact several amphibian and other vertebrates have their distribution restricted to areas south of the Doce River (Haddad et al., 2008). There is even an entire amphibian genus of forest-dependent direct-developing frogs (genus *Ischnocnema*) which is endemic to the southern part of AF (Canedo and Haddad *inpress*). This means that, either the RM alone cannot explain the current phylogeographical pattern of some AF species, or the paleomodeling of the AF indeed failed to predict large forested areas south of the Doce River.

Yet, if there were refuges in the south, where were they? Prace (1982) suggested that in the Serra do Mar slopes, at the coastal area between Sao Paulo and Rio de Janeiro states, continuous wet conditions over time could maintain large forest fragments. Behling (1997) also suggests that during the Last glacial Maxima the AF was restricted to a narrow belt between the coast and the Serra do Mar highlands. Stable areas should harbor high genetic diversity and are expected to be the center of origin of current lineages and species (Carnaval et al., 2009; Graham et al., 2006). Therefore the estimation of geographical origins of genealogies can be seen as an alternative way to identify refugial areas (Lemmon and Lemmon, 2008), with the advantage of not being restricted to a certain timeframe, as paleomodeling is. Furthermore, they are an objective way to check for congruence between paleomodels and genetic data. Nevertheless, to consistently characterize a refugium, centers of origins

of co-distributed species or lineages should overlap, indicating a common biogeographyc history and shared stable area among different species.

Following this idea, a phylogeographical analysis of eight AF amphibians and one snake species was performed. We generated data for three frog species (*Ischnocnema guentheri, I. parva and D. minutus*), whereas data from the other six species (*Hypsiboas faber, H. semilineatus, H. albomarginatus, Proceratophrys boiei, Rhinella crucifer* and *Bothrops jararaca*) were available on GenBank. To compare the phylogeographical pattern among the different species we applied a recently developed method that allows the analysis of genetic and geographic data simultaneously. The method can be used to estimate the origin of nodes and dispersal of genealogies, therefore it provides an objective way to compare phylogeographical patterns among species. To perform the analysis, well-sampled species or complex of species were chosen in order to provide enough genetic and geographic information for the phylogeographic reconstruction. The advantage of using amphibians in the phylogeographic analyses is their low tolerance to drought which is also a trait of the ombrophilous forests (Frankee et al., 2005; Haddad et al., 2008). Thus, it is expected that amphibian species would have imprinted in their genealogy the history of forest fragmentation and stable areas if they existed (Zeisset and Beebee, 2008). According to the RM: i) the geographical origin of co-distributed lineages have to overlap; ii) geographical origin of sister lineages have to be allopatric, away from contact zones; iii) consequently, the pattern will evidence that contact zones were established relatively later. Thereby, the present work intended to test whether the RM could be supported in the Brazilian AF using a newly developed phylogeographical method, and if a stable area south of the predicted one can be identified.

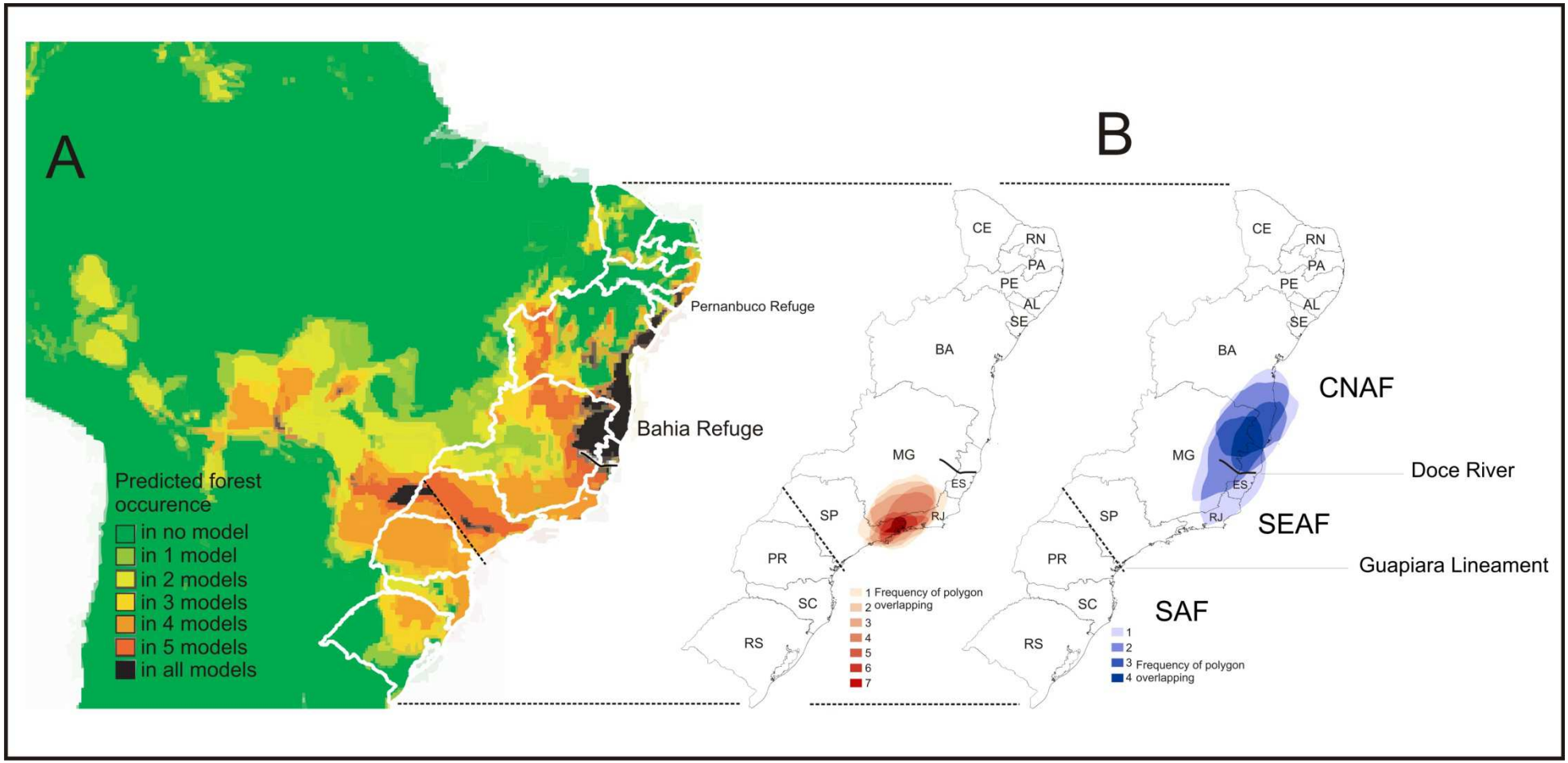

Figure 13:A) predicted AF refugia according to the paleoclimatic modeling performed by Carnaval and Moritz 2008 (image modified from Carnaval and Moritz 2008). B) Map of political borders o eastern Brazilian states with overlapping polygons of estimated center of origin. Dashed line in the map represents the Guapiara lineament and solid line the Doce River. Brazilian states abreviations: Rio Grande do Sul (RS); Santa Catarina (SC); Paraná (PR), Sao Paulo (SP); Rio de Janeiro (RJ); Espirito Santo (ES); Minas Gerais (MG); Bahia (BA); Sergipe (SE); Alagoas (AL); Pernabuco (PE); Paraiba (PA); Rio Grande do Norte (RN); Ceará (CE). Schematic subdivision of the AF: Center-Nortth AF (CNAF); Southeast (AF); South AF (SAF). Polygons of northern lineage of *H. albopunctatus*, southern lineage of *H.faber* and *B jararaca* are not included.

VII.II. Methods

VII.II.a. Data collection

To compare the geographical origin of co-distributed taxa, data of mitochondrial DNA sequences available on GenBank from the following five amphibian species or complex of species were downloaded: *Rhinella crucifer* complex(Thomé et al., 2010), *Proceratrophys boiei*(Amaro et al., 2012), *Hypsiboas faber*, *Hypsiboas albomarginatus* and *Hypsiboas semilineatus*(Carnaval et al., 2009). Data for other amphibian species generated within the present study were also used which are not yet available on GenBank , namely: the entire *Ischnocnema guentheri* complex (Gehara et al. unpublished; chapter one), the *I.parva* complex (Barth et al unpublished); and one Atlantic Forest lineage of *D. minutus* (lineage j) (Gehara et al. unpublished; chapter two). Additionally we analyzed data from the *Bothrops jararaca* complex (Grazziotin et al., 2006).This is a forest-dwelling snake and should also be a good indicator of forested stable areas. A summary of the data used with the description of the DNA fragments is available on **Table 4**.

Table 4: Species used in the phylogeographical analysis with respective molecular marker, substitution model, phylogeographical model and source.

Species	Marker	Substitution model	Phylogegraphical model	Source
Poceratroprys boiei	16S	TN+G	BD	Amaro et al 2012
	Cytb	TN+G		
Rhinella crucifer	ND2	TN+G	BD	Thomé et al 2010
	ND1	TN+G		
	Control Region	TN+G+I		
Bothrops Jararaca	Cytb	HKY+G	BD	Grazziotin et al 2006
Hypsiboas faber	ND2	TN+G	RRW	Carnaval et al 2009
Hypsiboas albomarginatus	ND2	TN+G	RRW	Carnaval et al 2009
Hypsiboas semilineatus	ND2	TN+G	BD	Carnaval et al 2009
Dendropsophus minutus	16S	HKY	BD	Chapter two
	COI	HKY		
Ischnocnema parva	16S	HKY+G	RRW	Barth et al unpublished
Ischnocnema guentheri	16S	GTR+G	RRW	Chapter one

VII.II.b. Phylogeographic analysis

Recently Lemey et al 2010, developed a Bayesian phylogeographical analysis to infer geographical origin of nodes and dispersal using ancestral reconstruction of continuous traits. The geographical coordinates of each sequence is used as traits and by reconstructing the traits at the nodes it is possible to reconstruct the biogeographical history of a particular genealogy. One advantage of the Bayesian implementation of the method is that topology uncertainty can be taken into account given that the ancestral traits and tree topology are estimated simultaneously. More specifically, the geographical origin of a certain node is estimated for the whole universe of high likelihood trees. Thus, using this approach is possible to calculate a probability distribution of the geographic origin of a certain node which can be represented as a polygon in a map. It is worth to clarify that that the polygons do not represent the ancestral distribution of the lineage or taxa, they actually represent a probability surface of the origin of a particular node, which is derived from the 80%HPD of the location of the node (estimated latitude plus estimated longitude). Because the method analyzes the association between genealogy and geography, it has the advantage of being suitable for comparative analyses even if different genes are used for each species.

The analyses were carried out in the software Beast 1.7.x. (Drummond et al., 2012).The best fit mutation model was calculated with jModel Test (Posada, 2008), using Akaike Information Criteria (AIC)(Akaike, 1974). For each species analyzed we assumed a lognormal relaxed clock model. Preliminary analysis showed that the posterior distribution of the standard deviation of the molecular clock (*ucld.stdv* parameter) does not include zero, which is an indication that the data significantly deviates from a strict molecular clock (McCormack et al., 2011). However, evaluation of node heights is beyond the scope of the present work, and the molecular clock model is not likely to influence the ancestral reconstruction of node traits. A coalescent prior with constant population size was assumed for all species. Mutation models used for each species are available on **Table 4**. When more than one mitochondrial gene per specie was used the alignment was partitioned in coding and non-coding regions to allow different substitution models and codon partitioning for the protein coding genes. As traits we used the geographic coordinates provided in the Supplementary Material of the original study that generated the data. A jittering code was used to generate random noise in case of equal geographical traits. The MCMC chain was ran long enough to ensure mixing of parameters and Effective Sample Size (ESS) values higher than 200,000. The program Tracer1.5 (Rambaut and Drummond, 2009) was used for monitoring the MCMC.

There are two available models for the phylogeographic reconstruction, the Homogeneous Brownian Diffusion model (BD), which assumes constant dispersal rate across branches, and the Relaxed Random Walk model (RRW), which allow variation of the dispersal rate across branches. For each species separate analyses were performed using each model. For the RRW a log normal prior distribution for the dispersal rate was used. To choose between the models a Bayes Factor comparison of marginal likelihood estimations of the root location was performed (Lemey et al., 2010). Despite the Bayes factor being considered not a good method for model fitting evaluation (Baele et al., 2012); it is currently the most appropriate method available in the Beast package. After choosing the best phylogeographical model, Maximum Credibility Trees (MCC) were summarized using Tree Anotator 1.7.x (Drummond et al., 2012), and inputted to the program SPREAD (Bielejec et al., 2011) which generates a *.kml* animation file of the phylogeographical reconstruction. The file is composed by a branching graphic which represents the tree and a group of polygons which represent the geographical origin of the nodes of the tree. As the aim of this study is evaluate the congruence among centers of origins of lineages and species we isolated the polygons correspondent to the nodes of interest. To do so, the *.kml* files were converted to shape files using an online based conversor (www.safe.com). Then, the polygons were isolated using MapWindow 4.8.6. Polygons of highly supported monophyletic sister clades that clearly represented northern and southern lineages, within a species or complex of species, were isolated. For species that a clear north-south discontinuity cannot be verified, the polygon of the origin of the entire species was isolated. To check for congruence, polygons of co-distributed lineages were overlapped.

VII.III. Results

In order to facilitate the discussion and understanding of the results we divide the AF in three regions. I) Center-North Atlantic Forest (CNAF) which correspond to the area north of Doce River; ii) the South East Alantic Forest (SEAF) which correspond to the area between Doce River and the Guapiara Lineament and associated Paranapanema River; and iii) South Atlantic Forest (SAF) which correspond to the areas south of Paranapanema River (**Figure 13**).

The results of Bayesian phylogeographical analyses showed high ESS and chain mixing in both models (BD and RRW) for most of the data. For some species, however, the RRW model showed poor mixing and low ESS while the BD showed high ESS values. Poor mixing can be caused by overparameterized models (see Beast discussion forum, *groups.google.com/group/beast-users*), therefore we assumed the BD as the best model in case of poor mixing of the RRW. A summary of models used for each dataset is presented in **Table 4**.

For some lineages and species the geographical origin coincided with the center of their distribution. To verify if the estimated polygon was not a result of a random phylogeographical association we shuffled the coordinates within the lineages and repeated the analysis. The resulted polygon from shuffled coordinates encompasses almost the entire distribution of the lineages (data not shown).

Following this result we considered that polygons generated with the real data that followed the same pattern resuted of random phylogeographical association. The only polygon the follow this pattern is the one relative to the northern lineage of *H. albomarginatus*. Thus, we considered that the geographical origin of this lineage cannot be recovered with this dataset.

For *Proceratrophys boiei*, and *Rhinella crucifer* and all *Hypsiboas* species paleomodelings are available and can be compared with the results presented here. For *P. boiei* the geographical origin of the species was estimated in a more restricted area than the predicted refuge for this species (see Amaro et al. 2012). For the *Rhinella crucifer* complex we found poor accordance between the phylogeographic reconstruction and the refugium modeled for this species (Thomé et al., 2010). For the all Hypsiboas species the estimated origin is in agreement with predicted refugiom. For *I.parva*, *I.guentheri*, *Bothrops jararaca* and *Dendropsophus minutus* paleomodeling is not available yet.

In general all phylogeographical reconstructions show that sister lineages originated in areas apart from each other and away from contact zones (**Figure 14, Figure 15, Figure 16**). Estimated geographical origins of northern lineages of *Hypsiboas faber*, *H. semilineatus;* and southern lineage of *H. albomarginatus* are located in the south of Bahia state and north of Espirito Santo state (**Figure 13, Figure 14**). The same area is showed as the center of origin of the northern lineage of *Rhinela crucifer* complex (**Figure 15**). The overlapping region of all these polygons support the Bahia Refuge proposed by Carnaval and Moritz (2008) (**Figure 13**). The northern lineage of *Bothrops jararaca*; and southern lineages *Rhinella crucifer* and *H. semillineatus* have their northernmost distribution south of the Bahia Refuge. These lineages have their geographical origin in the border region among Sao Paulo, Rio de Janeiro and Minas Gerais states. The same region was estimated as center of origin of species which are restricted to SEAF and SAF like *Ischnocnema guentheri*, *I. parva* and the *Dendropsophus minutus* (**Figure 15, Figure 16**). This area coincides with the Serra do Mar and Serra da Mantiqueira massifs. Two lineages have their distribution restricted two SAF, like the southern lineages of *Bothrops jararaca* and *H. faber* (**Figure 14, Figure 15**). Their estimated centers of origin overlap in the SAF. However, because of the small number of species analyzed for this region we decided not to take conclusions about this region and not to include centers of origins of SAF lineages in the overlapping procedure.

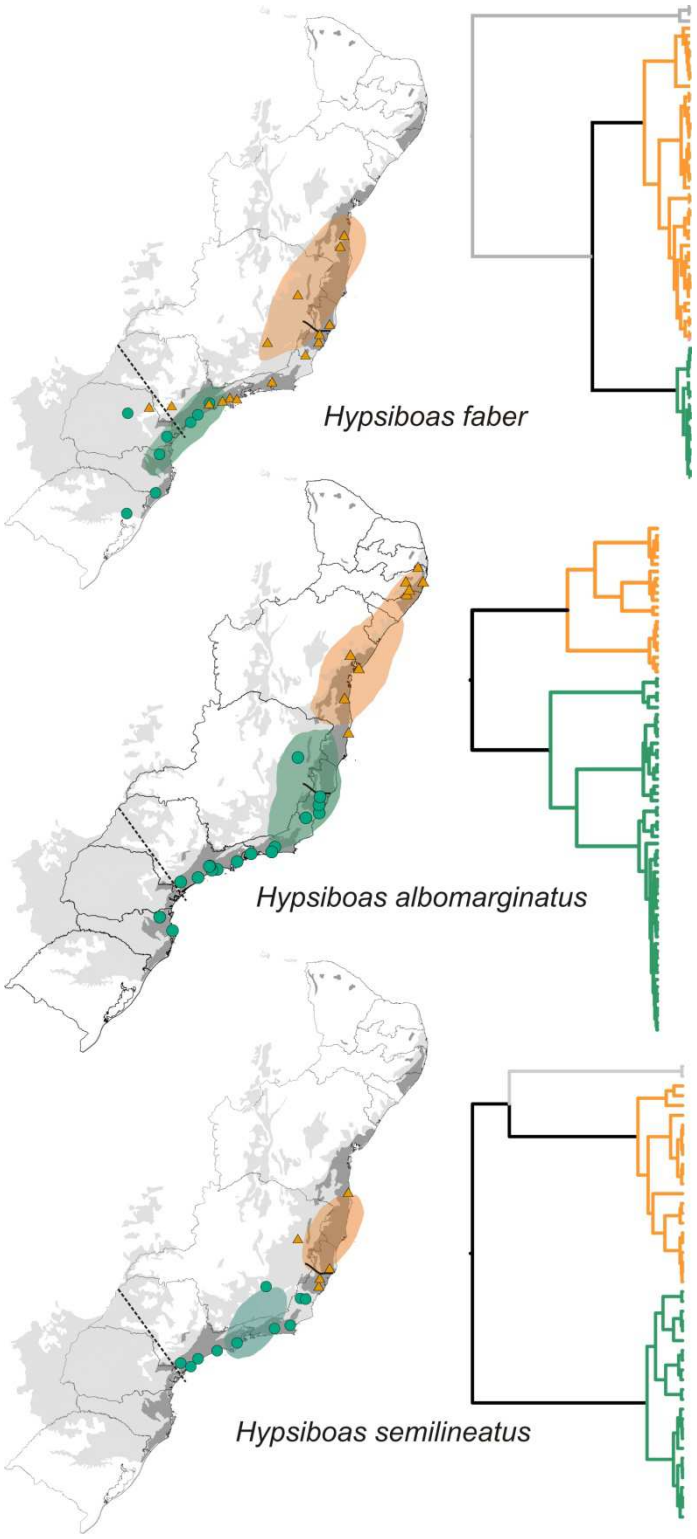

Figure 14: Map of eastern Brazil with political borders of states. Distribution of lineages of three *Hypsiboas* species with estimated centers of origin. Northern lineages are respresented in orange, southern lineages are represented in green. Dark grey represent the ombrophilous forest, light grey respresent other AF biomes including semi-deciduous forest and araucaria forest

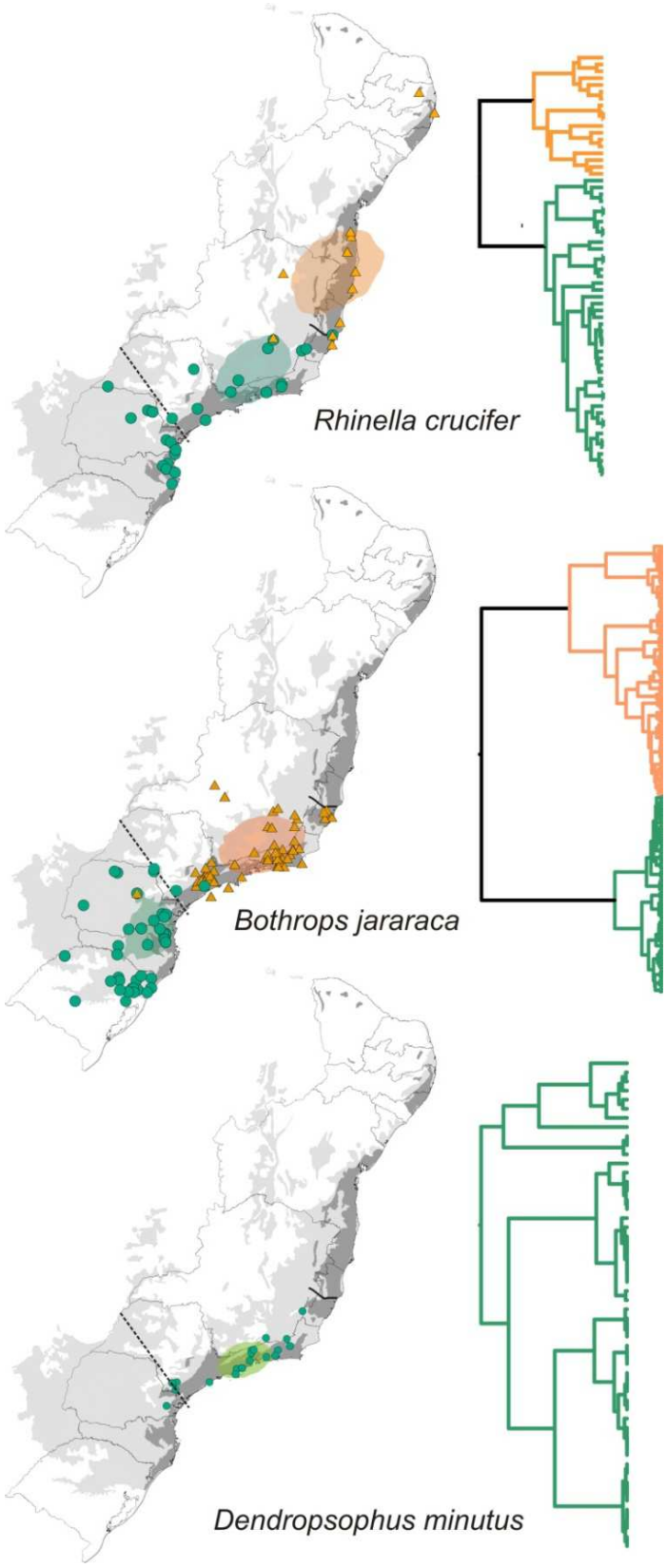

Figure 15:.Map of eastern Brazil with political borders of states. Distribution of lineages of *Rhinela crucifer*, B*othrops jararaca* and *Dendropsophus minutus* with estimated centers of origin. Northern lineages are respresented in orange, southern lineages are represented in green. Dark grey represent the ombrophilous forest, light grey respresent other AF biomes including semi-deciduous forest and araucaria forest

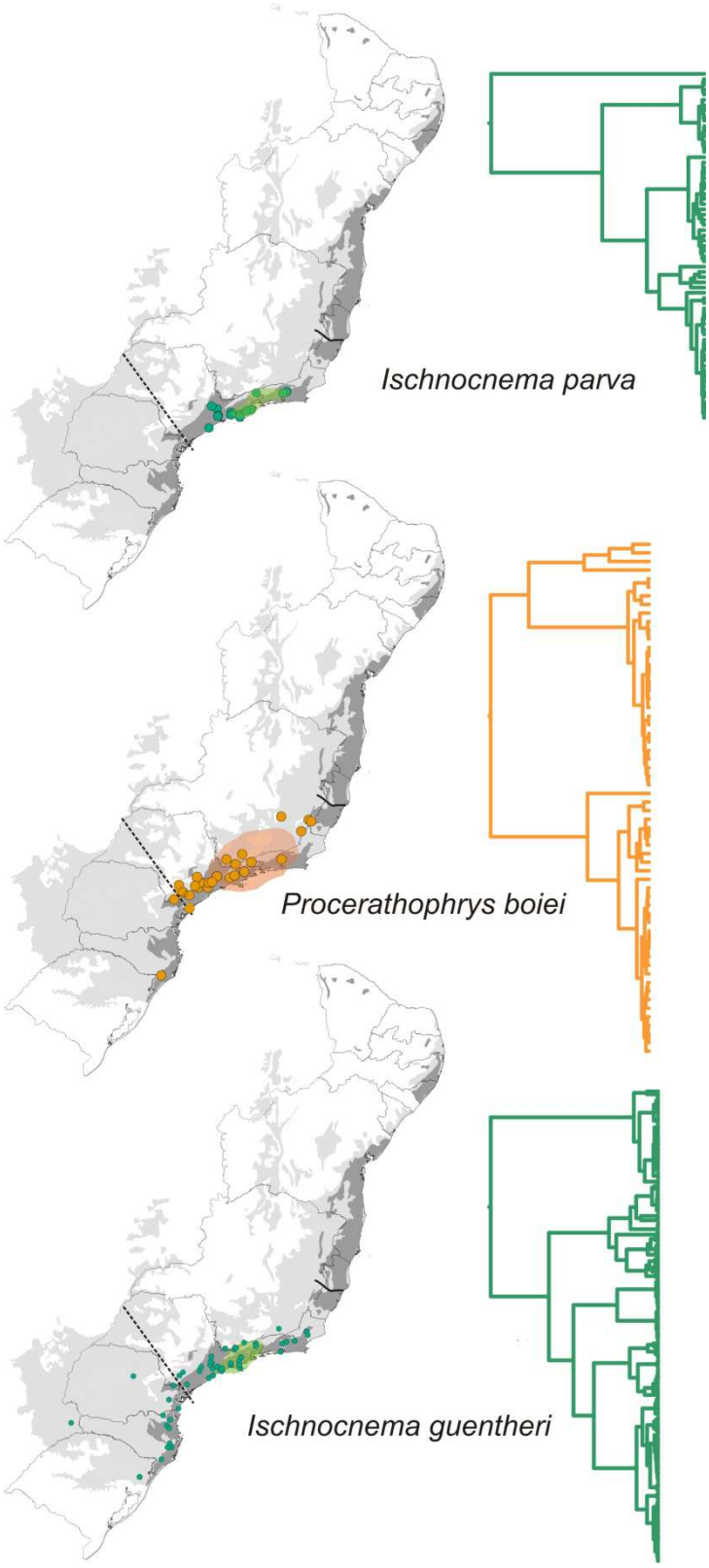

Figure 16:.Map of eastern Brazil with political borders of states. Distribution of *Ischnocnema parva* (green), *P. boiei* (orange) and *I. guentheri* (green) with estimated centers of origin. Dark grey represent the ombrophilous forest, light grey respresent other AF biomes including semi-deciduous forest and araucaria forest

VII.IV. Discussion

In this study we compared phylogeographical patterns among nine co-distributed AF species. The approach employed for the comparison is based on a dispersal model that simply analyzes the phylogeographical history of a genealogy in the landscape by estimated the geographical origin of nodes. Considering the coalescence process, the origin of a certain node in a genealogy could be the result of a stochastic process. However, the congruency between different species and genealogies makes very unlikely that the patterns found were randomly generated. The resulting phylogeographical reconstruction demonstrates that sister lineages originated away from contact zones and away from one another, suggesting allopatric diversification and recent contact between lineages. It is striking the overlapping of estimated geographical origins of co-distributed taxa and lineages, which is in agreement with the existence of a common biogeographical history and with a diversification center. This pattern support the RM and is in agreement with recent studies that suggest climatic oscillations as an important factor on diversification of AF biota (Carnaval et al., 2009; Carnaval and Moritz, 2008; d'Horta et al., 2011). The overlapping of center of origins, one in the CNAF and another at the SEAF, allow the identification of two diversification centers in AF.

VII.IV.a. The CNAF diversification center

The work of Carnaval et al (2009) demonstrated that stable areas have maintained most of the genetic diversity of three frog species while unstable areas were recently colonized. The presence of overlapping center of origins at the region of southern Bahia and northern Espirito Santo state (**Figure 13**) is in agreement with the Bahia Refugium (Carnaval and Moritz, 2008). Moreover our results show a later dispersion to the south for the three *Hypsiboas* species. However, while northern lineages of *H. semilineatus* and *H. faber* originated in the Bahia Refugium, the genealogical pattern and the philogeographical reconstructions suggest that southern lineages originated south of this area, in the SEAF. Although the paleomodeling of the AF suggested the absence of large stable areas in the SEAF (Carnaval and Moritz, 2008), Carnaval et al (2009) considered two stable areas for *H. faber* and *H. semilineatus*, one in CNAF and another in SEAF (see supplementary methods of Carnaval et al. 2009). The results presented here provide additional evidence to support a stable area in SEAF.

VII.IV.b. The SEAF diversification center

For most of the species and lineages distributed in SEAF, the estimated areas of origin overlap at the boundary area between Sao Paulo and Rio de Janeiro (**Figure 13 B**). This area was previously suggested as being stable during climatic oscillations (Behling, 1997; Prance, 1982). Not surprisingly, the overlapping polygons in center AF encompass two mountainous formations, the Serra da

Mantiqueira and the Serra do Mar massifs. The existence of an altitudinal gradient provides opportunity for vertical migration of the forest and associated fauna during climatic oscillations. At high elevations forest continuity and high humidity conditions could be maintained when the lowlands were unsuitable, forming islands of forest on the top of mountains. A similar scenario has been demonstrated for the Autralian wet tropics (Graham et al., 2006). Aditionally, altitudinal gradient would generate strong environmental gradient increasing the chance of adaptation. Thus, topographical heterogeneity would increase the opportunity for speciation and at the same time maintain diversified lineages or species (Graham et al., 2006; Moritz et al., 2000; Smith et al., 2007; Wollenberg et al., 2008). Some of the species analyzed here are typical high elevation species (*I. guentheri*, *I. parva*, *P. boiei*); some occur at both high and low elevations (*B. jararaca*, *D. minutus*, *H. faber*, *R. crucifer*). Populations of these species could be maintained at high or middle elevations if the lowlands became unsuitable.

The results of *I. guentheri* and *I. parva* are particularly interesting. These are two complex of species (see Chapter 1, Barth et al unpublished data) of leaf-litter frogs highly dependent of forest and humidity. The resulting areas of origin of the respective mitochondrial lineages are very small and encompass mainly mountainous regions. Our analysis suggested that for both species all deep mitochondrial lineages have originated within the polygon area and that some lineages subsequently dispersed to the south. As suggested by Amaro et al. (2012) high land species could experience an expansion of their distribution during cold periods, given that those species are more adapted to lower temperatures. Then, it is also possible that these species migrate to the south during glaciations periods if humidity conditions were fulfilled. For other species or complex of species such as *B. jararaca* complex, *D. minutus*, *H. semilineatus*, *R. crucifer* complex the same area corresponds to the origin of one of the sister mitochondrial lineages. The mitochondrial lineages of these taxa have likely diversified by vicariance when populations of these species became isolated in stable areas. From these stable regions the species dispersed to other areas, getting in contact and forming hybrid zones in some cases. For instance, a similar hybrid zone exists between species of the *R. crucifer* complex and between *Phylomedusa burmeisteri* and *P. baiana*, both around the area of Doce River (Brunes et al., 2010; Thomé et al., 2010). In this case rivers and faults would act as secondary barriers, decreasing gene flow between diversified lineages, but not as the main source of divergence. Rivers were also suggested to be secondary barriers for AF birds populations that likely diversified by vicariance (Cabanne et al., 2008; d'Horta et al., 2011).

Recently, Silva et al (2012) proposed a biogeographical region limited by the Rio Doce River and the Guapiara Lineament, which they called the South East vertebrate component (Silva et al., 2012). Their analysis was based on previous diversity and endemism analysis of vertebrates (Cardoso da Silva et al., 2004; Costa et al., 2000), but analysis of plant diversity also support their proposal (Prance, 1982). Besides the South East vertebrate component and other proposed components for

northern areas of AF, Silva et al propose another biogeographical component south of Guapiara lineament. Unfortunately, from the species analyzed in our work, the only broadly-sampled in SAF is *B. jararaca*. Interestingly, the center of origin of its southern lineage also encompasses a mountainous area. Future analysis of species distributed in southern AF are required to verify the existence of a common center of origin in this area. Although our results support vicariant diversification between refuges, sympatric or parapatric diversification within stable regions cannot be excluded. For *H. albomarginatus* the center of origin of both lineages are next to each other and within a predicted stable region. The same occurs in *I. guerntheri* and *I. parva*. For *I. guentheri*, only two species of the complex were able to disperse to the south (see chapter one). It is possible that species within this complex have adapted to different ecological conditions. Further investigation is needed to verify this hypothesis. The center of origin of the southern lineage of *H. faber* contrast with the pattern found here for the other species given that it was located between the overlapping area at center AF and the center of origin of the southern *B. jararaca* lineage. This can be an artifact caused by the rather restricted sampling for this lineage which would result in insufficient geographic and genetic information for the identification of its center of origin. Nevertheless, an origin at this area is possible given the flexibility of this species in occupying a broad variety of habitats within the AF

VII.IV.c. Time of diversification

One possible criticism to the conclusions taken here is that the diversification of those lineages may not have occurred simultaneously. Indeed, the approach used here considers only the relative time of diversifications within each species. In one hand this impedes us to determine the simultaneous occurrence of diversifications, but on the other hand the analysis performed here has the potential to reveal areas that were important for diversification throughout the time. Therefore we acknowledge that it is possible that those diversification events happened at different times. However, evidence from another study support simultaneous divergence between mitochondrial lineages of *R. crucifer* complex, *H. faber*, *H. semilineatus*, *H. albomarginatus*and *B. jararaca* complex (De Mello Martins, 2011). Our results complement this idea by demonstrating that most of the sister mitochondrial lineages have a common center of origin. Therefore it is not unreasonable to speculate that those lineages diversified in the same event. Nevertheless, the diversifications of *I. guentheri and I. parva* complexes, *D. minutus* AF lineage and *P. boiei* were not included in the analysis performed by De Mello Martins (2011) and the divergences within these groups could represent much older periods.

In most phylogeographical studies of AF fauna, evolutionary biologists use molecular dating of population divergence and demographic changes to frame these evolutionary processes within paleogeological periods. These estimates are used to bear the arguments to reject or to propose a diversification hypothesis. For instance, divergences that cannot be correlated with climatic

oscillations, and population expansions that do not coincide with Holocenic warming can be seen as evidence to reject the RM. A common trend found among these estimates is an early divergence dating from Pliocene-Pleistocene transition, sometimes even Miocene; and population expansion that predate the Holocenic warming (Amaro et al., 2012; Fitzpatrick et al., 2009; Grazziotin et al., 2006; Pellegrino et al., 2005; Thomé et al., 2010). Evidence from the literature support the idea that the divergence times and dating of population expansions calculated for some AF species are likely overestimated (Grant et al., 2012; Ho et al., 2011; Ho et al., 2005). Two possible caveats that can cause these overestimations can be highlighted:

1) Molecular clock calibration: because of the lack of recent fossils for molecular clock calibrations, researchers have to calibrate deep nodes and/or employ substitution rates derived from those deep calibrations to date relatively recent events. The molecular clock calibrations of deep nodes generate an underestimation of the spontaneous mutation rate that happen at population level (Ho et al., 2011). It has been observed that the mutation rate reduces exponentially towards the past (Ho et al., 2011; Ho et al., 2005), therefore, the older the calibration is, the higher will be the overestimation of recent node heights.
2) Dating Approach: divergence datings of AF species are commonly based on mitochondrial DNA only. If ancestral population was large (ancestral polymorphism) and the divergence of species happened with reduction in population size, which would be expected considering the RM, the estimated time of the most common ancestor between these populations will very likely pre-date the real time of divergence (Jennings and Edwards, 2005). The inverse relationship between coalescence probability and population size implies that the larger the ancestral population was, the higher is the chance to have a deeper coalescence among diverged populations and consequently higher is the chance of an overestimation of the divergence, particularly when it is based on the time to the most recent common ancestor of a particular genealogy. Consequently, divergence dating methods that take into account population parameters yield contrasting results when compared to phylogenetic methods (see differences between divergence estimates of Grazziotin et al 2006, Thomé et al 2010; and De Mello Martins 2011).

Thus, molecular dating of shallow nodes or recent events has to be critically revised. The use of reliable mutation rate and multi-locus analysis is needed in order to have a reliable estimate of time divergence of populations within species and among closely related species (Jennings and Edwards, 2005).

VIII. Outlook

In this thesis I presented the molecular analysis and biogeography of neotropical frogs. The results presented in chapters one and two provide additional evidence to support the idea that the number of species in the neoptropics is largely underestimated. Also, the degree of microendemism of direct developing frogs in the AF might be higher than previously though. In the first chapter we found that *Ischnocnema guentheri* is endemic to its type locality, Rio de Janeiro, evidencing the importance of urban forests for conservation of biodiversity. In the second chapter we identified an interesting biogeographical pattern for *D. minutus* with recurrent dispersals between Amazonia and Atlantic Forest. In the third chapter a comparative analysis of Atlantic Forest species was performed. Despite the preliminary character of this chapter, the results are interesting showing a striking overlapping pattern of centers of origin of mitochondrial lineages of Atlantic Forest species. Interesting remarks can be considered based on the results presented here.

Regarding chapter one, additional work will be needed to better define each species of the *Ischnocnema guentheri* complex. Morphological analysis will be necessary to confirm each candidate species and support description of each of them. We intend to work on a more comprehensive taxonomic revision of the complex soon.. Additional samples of unanalyzed localities are already available; two additional nuclear genes were already sequenced and will be included in a more comprehensive analysis. I plan to use a population genetics approach for the identification of populations (Weisrock et al., 2010), which can be used for helping in the identification of non monophyletic groups. A complete phylogeographic analysis of the complex will also be performed.

For a proper taxonomic revision of the *Dendropsophus minutus* group other types of data are needed. Advertisement call variation and morphology have to be included in order to define the number of existent species within the group. Nuclear data should also be included to check if the mitochondrial phylogeny can be supported by other markers and to check for evidence of migration. It is possible that other markers reveal a biogeographical pattern different of the one found using the mitochondrial markers. However it is clear that there are several species being called *D.minutus* and that the group originated in the Amazonian area while the *D. minutus* complex has its origin in the Atlantic Forest.

In chapter three, the preliminary analyses allow us to better delineate further improvements. For instance, the generation of paleomodeling distributions of all the species analyzed will be performed using the same methodology, so they can be compared with the molecular estimation of centers of origins. It would be also important to use new methods of model fitting to evaluate which phylogeographical model best fit the data. Newer versions of Beast will likely have implemented in

their codes the Path Sampling and the Stepping Stone approach of model evaluation, which are shown to be more appropriate when compared with marginal likelihood bayes factor (Baele et al., 2012).

In conclusion, this thesis adds important information to help in the understanding of tropical diversity and the diversification processes involved. It helps improving the taxonomic knowledge about neotropical frogs by opening a wide door for more complete studies of *Ischnocnema guentheri* and *Dendropsophus minutus* species complex. It brings new ideas about the South America biogeography as well as diversification processes within the Brazilian Atlantic Forest. By improving our knowledge about biological information it consequently influences future conservation measures, providing new information to be taken into account (like species range, genetic diversity and diversification centers). It is however, a small brick added in the wall of knowledge. But,

"as *the builders say, the larger stones do not lie well without the lesser*" (Plato)

IX. References

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Werneck, F.P., Nogueira, C., Colli, G.R., Sites, J.W., Costa, G.C., 2012b. Climatic stability in the Brazilian Cerrado: implications for biogeographical connections of South American savannas, species richness and conservation in a biodiversity hotspot. Journal of Biogeography, no-no.

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Wollenberg, K.C., Vieites, D.R., van der Meijden, A., Glaw, F., Cannatella, D.C., Vences, M., 2008. Patterns of endemism and species richness in Malagasy cophyline frogs support a key role of mountainous areas for speciation. Evolution 62, 1890-1907.

Wuster, W., Ferguson, J.E., Quijada-Mascarenas, J.A., Pook, C.E., Salomao Mda, G., Thorpe, R.S., 2005. Tracing an invasion: landbridges, refugia, and the phylogeography of the Neotropical rattlesnake (Serpentes: Viperidae: Crotalus durissus). Mol Ecol 14, 1095-1108.

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Marcelo Coelho Miguel Gehara

Curriculum Vitae

October, 2012

Curriculum Vitae

Full name: Marcelo Coelho Miguel Gehara

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Mother: Guilhermina R. C. Gehara

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Formal Education

2009 – present PhD candidate, Evolutionary Biology, Technical University of Braunschweig. Thesis: Molecular analysis and phylogeography of neotropical amphibians.
Advisor: Miguel Vences
Scholarship: Katolischer Akademisher Ausländer-Dienst

2007 – 2009 Master in Zoology
Pontifícia Universidade Católica do Rio Grande do Sul, PUC RS, Porto Alegre, Brazil
Title: Phylogeography of the Southern Sea Lion, *Otaria byronia* (Mammalia, Carnivora, Otariidae).
Advisor: Sandro Luiz Bonatto
Scholarship: Conselho Nacional de Desenvolvimento Científico e

Tecnológico, CNPq

2001 – 2005 Graduate in Biological Sciences
Universidade Federal de Juiz de Fora, UFJF, Juiz De Fora, Brazil
Advisor: Artur Andriolo

2000 – 2001 High School: 3rd year at "Opção Vestibulares" School

1998 – 2000 High School: 1st and 2nd years at "Stella Matutina" School

1986 – 1997 School at: Colegio Cristo Redentor – Academia de Comércio

Complementary Education

2008 – 2008 Short Term Course: Sailing for adults (50 hours)
Escola de Vela Barra Limpa – Clube dos Jangadeiros, Porto Alegre, RS, Brazil

2008 – 2008 Short Term Course in Conservation Biology of Fur Seals and Sea lions (16 hours)
Grupo de Estudos de Mamíferos Aquáticos do Rio Grande do Sul, GEMARS, Porto Alegre, RS, Brazil

2004 – 2004 Short Term Course in Neotropical Mastozoology. (7.5 hours)
Universidade de Brasília, UNB, Brasília, Brazil

2003 – 2003 Short Term Course in Animal Welfare. (20 hours)
Universidade Federal de Juiz de Fora, UFJF, Juiz De Fora, Brazil

2003 – 2003 Short Term Course in Macroecology of Neotropical Mammals. (5 hours)
Pontifícia Universidade Católica de Minas Gerais, PUC Minas, Belo Horizonte, Brazil

2003 – 2003 Short Term Course: Topics in biogeography of vascular plants. (20 hours)
Universidade Federal de Juiz de Fora, UFJF, Juiz De Fora, Brazil

2002 – 2002 Short Term Course in Pollution and Contamination of Hydric resources. (20 hours)
Universidade Federal de Juiz de Fora, UFJF, Juiz De Fora, Brazil

2002 – 2002 Short Term Course in Conservation Biology. (12 hours)
Universidade Federal de Juiz de Fora, UFJF, Juiz De Fora, Brazil

2001 – 2001 Short Term Course in Tropical and Sub-tropical forests. (8 hours)
Universidade Federal de Juiz de Fora, UFJF, Juiz De Fora, Brazil

2001 – 2001 Short Term Course in Zoological Illustrations. (20 hours)
Universidade Federal de Juiz de Fora, UFJF, Juiz De Fora, Brazil

Professional experience

Universidade Federal de Juiz de Fora – UFJF

2004 – 2005 Contract: exhibitioner
Position: trainee
Working hours (weekly): 20, full time exclusiveness
Activities: Scientific Initiation, Scholarship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)
Research Project: Popular Knowledge of the genus *Mazama* at the surroundings of the Ibitipoca State Park, MG, Brazil.

2002 – 2003 Contract: Collaborator
Position: trainee
Working hours (weekly):12
Activities: apprenticeship
Research Project: Entomological Fauna of the Santa Candida Municipal Forest Reserve, Juiz de Fora, MG, Brazil

2002 – 2003 Contract: exhibitioner
Position: trainee
Working hours (weekly): 12
Activities: Teaching, scholarship from Universidade Fedaral de Juiz de Fora – UFJF
Project: Teaching Science and Zoology, integrating schools and UFJF Zoology department

Macro Ambiente Tecnologia Ambiental – MATA

2003 – 2003 Contract: exhibitioner
Position: trainee
Working hours (weekly):20
Activities: Environmental consulting for *Vista Alegre* Farm, MG, Brazil, 350 hectares.

Areas

Zoology
Herpetology
Phylogeography
Phylogenetics
Population Genetics

Languages

Portuguese	Understands, Reads, Speaks, Writes
English	Understands, Reads, Speaks, Writes
Spanish	Understands, Reads, Speaks
German	Understands

Awards

2006 Section award at XII Seminário de Iniciação científica da UFJF: (Oral presentation) Conhecimento popular no estudo de Cervídeos do Parque Estadual do Ibitipoca e no seu entorno, MG. 2006. In: XII Seminário de Iniciação científica da UFJF, Brazil.

Papers Published in Scientific Journals

Fonseca, M; Carvalho, RMH; Lanna, F; **GEHARA, M**.
Predation on *Sibynomorphus neuwiedi* (Serpentes: Dipsadidae) by *Leptodactylus labyrinthicus* (Anura: Leptodactylidae) in southeastern Brazil. Herpetology Notes, v. 5, p. 167-168, **2012**.

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Participation/Publication in events (Abstracts)

Canedo, C; **GEHARA, M**; Vences, M; HADDAD, CFB
Molecular and acoustic analyses of *Ischnocnema guentheri* species complex (Anura: Brachycephalidae). In: IX Congresso Latinoamericano de Herpetologia, **2011**. Resumos do IX Congresso Latinoamericano de Herpetologia, 2011, Curitiba, Brazil.

Hauswaldt, JS; Angelini, C; Benavides, E; **GEHARA, M**; Polok, A; Steifartz, S
The genus *Salamandrina* (spectacled salamanders) from Italy: phylogeography and population genetics. In: SHE European Congress of Herpetology. **2011**. Luxembourg

GEHARA, M; Canedo, C; Haddad, C; Vences, M
Molecular analysis of *Ischnocnema guentheri* highlights a complex of cryptic species. In: XI Congreso Luso-Espanol / XV Congreso Espanol de Herpetología. **2010**. Sevilla, Spain.

GEHARA, M; Oliveira, LR; Majluf, P; Cárdnas, SA; Pavés, HJ; Bonatto, SL
(Oral presentation) - Filogeografia do Leão-Marinho-do-Sul, *Otaria byronia* (OTARIIDAE): Uma comparação entre as populações dos oceanos Atlântico e Pacífico. In: XIII Reunión de Trabajo de Especialistas en Mamíferos Acuáticos de América del Sur, **2008**, Montevideo, Uruguay.
"Phylogeography of the Southern Sea Lion, Otaria byronia (OTARIIDAE): Comparing the populations of the Atlantic and Pacific oceans"

GEHARA, M; Oliveira, LR; Majluf, P; Cárdnas, SA; Bonatto, SL
Diversidade genética, estruturação populacional e status taxonômico do Leão-Marinho-do-Sul, *Otaria byronia* (Otariidae). In: 53^{O} Congresso Brasileiro de Genética, **2007**, Águas de Lindóia, SP.
"*Genetic Diversity, population structure and taxonomic status of the Southern sea lion*"

GEHARA, MCM; RIBEIRO, GC; BISAGGIO, EL; ANDRIOLO, A.
(Oral pesentation) - Conhecimento popular no estudo de Cervídeos do Parque Estadual do Ibitipoca e no seu entorno, MG. **2006**. In: XII Seminário de Iniciação científica da UFJF, Brazil.
"Popular Knowledge of the genus Mazama at the Surroundings of the Ibitipoca State Park"

GEHARA, M; Bisaggio, EL; Ribeiro, GC; Andriolo, A
Popular Knowledge of the genus *Mazama* at the Surroundings of the Ibitipoca State Park, MG In: AnnualMeeting of the Association for Tropical Biology And Conservation, **2005**, Uberlândia, Brazil.

Ribeiro, GC; **GEHARA, M;** Bisaggio EL; Andriolo, A
Sightings of *Mazama* by local community at the surroundings of the Ibitipoca State Park, MG In: AnnualMeeting of the Association for Tropical Biology And Conservation, **2005**, Uberlândia, Brazil.

Bisaggio, EL; Ribeiro, GC; **GEHARA, M;** Andriolo, A
Vegetation composition and the occurrence of *Mazama* in the State Park of Ibitipoca and Surroundings, MG In: Annual Meeting of the Association for Tropical Biology and Conservation, **2005**, Uberlândia, Brazil.

Mathias, AA; **GEHARA, M**; Martins, GN; Andriolo, A
Análisa Preliminar dos atropelamentos de mamíferos na ES-060, Espirito Santo. In: XXV Congresso Brasileiro de Zoologia, **2004**, Brasília.
"Preliminary analyses of the mammals dead caused by car run over on the ES-060 highway"

Brugiolo, SSS; Rezende, LS; Costa, RC; **GEHARA, MCM**; Barbosa, JM
Diversidade de Artropodos da Reserva Minicipal Santa Cândida, Juiz de Fora, MG. In: XXV Congresso Brasileiro de Zoologia, **2004**, Brasilia.
"*Arthropods Diversity of the Santa Candida Municipal Forest Reserve*"

Bisaggio, EL; **GEHARA, M**; Ribeiro, GC; Andriolo, A
Uso de armadilhas fotográficas no estudo de aspectos ecológicos e comportamentais do *Mazama* SP no Parque Estadual de Ibitipoca e seu entorno: Resultados Preliminares. In: XI Seminário de Iniciação Científica da UFJF, **2004**, Juiz de Fora, MG.
"*Using camera traps to study ecology and behavior of the Mazama sp in the Ibitipoca State Park and surroundings: preliminary results*"

GEHARA, MCM; Andrade, AJ; Romão, CR, Brugiolo, SSS

Levantamento da fauna entomológica da reserva biológica municipal Santa Cândida, Juiz de Fora, MG In: XXVI Semana de Biologia, **2003**, Juiz de Fora, MG "*Entomological fauna of the Santa Cândida Municipal Forest Reserve*"

Rezende, LS; **GEHARA, MCM**; Lima, SS
Dinamização do ensino de Ciências e de Zoologia. Integração Escolas e Departamento de Zoologia In: V seminário de extenção da UFJF, **2003**, Juiz de Fora, MG.
"*Teaching Science and Zoology, integrating schools and UFJF Zoology department"*

Andrade, AJ; **GEHARA, M**; Costa, RC; Romão, RC, Brugiolo, SSS
Reserva Muinicipal Santa Cândida, Juiz de Fora/MG: Uma Amostragem da Artropodofauna In: IV Encontro Nacional de Biólogos, **2002**, Ouro Preto, MG.
"Santa Candida Municipal Forest Reserve: An Arthropod Sampling"

Other Activities

1992 – 2000 Choir Singer in: Meninos Cantores da Academia – "Mater Verbi" Choir